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The Production of Hemorrhage in Sarcoma 180 in Mice by Anaphylaxis and the Inhibitory Effect of Cortisone on the Hemorrhage-Producing Factor from *E. coli*[1,2]

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Studies on the mechanism of the pyrogenic action of bacterial filtrates (*1*, *2*) led to the suggestion that hypersensitivity probably played an important role in the reaction. It was assumed that sensitization was brought about under normal conditions by adsorption of bacterial products through the intestinal mucosa. It follows then, that the hemorrhagic action of *Escherichia coli* filtrates on transplantable tumors (*3*, *4*) might also be the result of a hypersensitivity mechanism. In fact, Barrett (*5*) produced hemorrhage in Sarcoma 37 in 55 of 60 mice by the injection of horse-serum antigen into homologously sensitized mice after transplantation of the tumor.

The present study utilized crystalline bovine albumin as an antigen, and both active and passive types of sensitization. Since the tests showed that hemorrhage was produced in tumors following anaphylactic shock, it was of interest to include a series of tests on the effect of cortisone on activity of the tumor hemorrhagic factor (THF) from *E. coli* because of the possibility of an anaphylactic mechanism being involved. No studies were made on the effect of cortisone on known anaphylactic reactions.

Materials and Methods

Antigen and antiserums.—The antigen was a preparation of Armour's crystallized bovine serum albumin (BSA). Test solutions were always made up in pyrogen-free saline and under pyrogen-free conditions. The antiserums used for passive sensitization experiments were from rabbits and mice that had been immunized against BSA. The rabbit antiserum contained 1.76 mg. of specific precipitable antibody per ml. The mouse antiserum was not quantitatively analyzed for precipitable antibody but the pool used for passive transfer gave a strong positive ring-test.

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[2] This work was supported by a grant from the National Cancer Institute of the National Institutes of Health of the U. S. Public Health Service.

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[4] The authors wish to thank Professor Carl Niemann for his interest and encouragement in this work and Betty Whisenand for her technical assistance.

[5] Contribution No. 1937.

[6] The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

It was obtained from a group of 5-week-old mice that had received 3 intraperitoneal injections of 5 mg. of BSA on alternate days. Blood was obtained 12 days after the last injection.

Tumor hemorrhagic tests and anaphylaxis.—The tumor used was Crocker mouse Sarcoma 180 and the test animals were Marsh Buffalo white mice. (Both the tumor and the mice were of the same strain and colony used by Ikawa *et al.* (*4*) in their studies of THF from *E. coli.*) Unless otherwise noted, tumor transplants were inoculated into 6-week-old mice. Transplants consisted of small pieces of healthy tumor tissue introduced into the subcutaneous tissue of the lateral abdominal wall by means of a 17-gauge trocar. Tests were made 1 week later on a selected group, which showed healthy (no apparent hemorrhages or necrotic areas) tumors of about 7-mm. diameter. A positive hemorrhage reaction was one that occurred within 24 hours and involved at least 50 percent of the tumor surface. In most instances, the controls were free of any hemorrhage. Negative reactions in treated animals presented a problem in evaluation at times, but usually when a hemorrhage was induced, it involved at least 50 percent of the tumor surface at 24 hours.

Anaphylaxis was characterized by prostration and ruffled fur which persisted for several hours. Death usually occurred in 1 to 2 hours and animals that survived were apparently normal the following day. There is no doubt that the shock reaction was the result of a hypersensitivity to BSA, and passive transfer of this hypersensitivity by antiserum was strong evidence for an antibody mechanism. Although the reaction was not so violent as in guinea pigs, it paralleled gross reactions often seen in dogs or rabbits.

Active sensitization.—The results of experiments on active sensitization are summarized in table 1. Individual experiments usually consisted of approximately 10 mice per group, except for the sensitized and shocked group in which the number was increased in order to have a significant number of survivals. Animals were sensitized during their 5th week of age by giving them 3 injections of 2.5 mg. of BSA on alternate days. They were then inoculated with tumor tissue in the 6th week. Seven days after the tumor inoculation and 10 days after the last sensitizing injection, 2.5 mg. of BSA was injected intraperitoneally. Approximately 45 percent of the mice that survived anaphylactic shock developed tumor hemorrhages that involved at least 50 percent of the tumor surface. Group V has been included because of interest in the possibility that the reaction in tumors might have contributed to the high fatal anaphylaxis. However, the 60-percent deaths in the non-tumor group was actually higher than in the tumor group. The percent of hemorrhages produced by anaphylaxis was not so high as previously reported by Barrett; but this may be a reflection of the tumor strain, the antigen, or the quantitative relationships of antigen and antibody involved in the two studies. Regardless of the basic mechanism responsible for hemorrhage there is no doubt that the hemorrhagic reaction can be induced by anaphylaxis. In general, local hypersensitivity reactions are characterized by intense

infiltration of white cells and macrophages, but there was no evidence of such an inflammation in the tumor hemorrhages produced in the present studies. A fall in blood pressure, which is characteristic of anaphylaxis in most animals, might produce a temporary but significant anoxia as suggested by Algire (*6*).

TABLE 1.—*Summary of results on the tumor hemorrhagic effect of anaphylaxis in actively sensitized mice*

Group	Antigen (BSA)	Tumor	Antigen (BSA)	Total mice	Survivors (*number*)	Survivors showing hemorrhage in tumors (*number*)	Survivors showing hemorrhage in tumors (*percent*)
I	0	+	0	58	58	1	1. 7
II	+	+	0	40	40	2	5. 0
III	0	+	+	39	39	2	5. 1
IV	+	+	+	90	53	24	45. 4
V	+	0	+	20	8	—	—

Passive sensitization.—The results of passive sensitization and shock have been summarized in table 2. Data dealing with attempts to passively sensitize with rabbit anti-BSA serum have not been included since these results were uniformly negative and produced no evidence of anaphylaxis or hemorrhagic reactions. The failure of rabbit antiserum to sensitize mice passively was not entirely unexpected since heterologous serums often fail to do this. However, the homologous mouse antiserum did produce mild sensitization, and, although the injection of antigens

TABLE 2.—*Summary of results on the tumor hemorrhagic effect of anaphylaxis in mice passively sensitized with mouse anti-BSA serum*

Group	Tumor	Antiserum to BSA	Antigen	Total mice	Deaths	Mice showing anaphylaxis (*number*)	Mice showing hemorrhages in tumors (*number*)	Mice showing hemorrhages in tumors (*percent*)
I	+	0	0	10	0	0	1	10
II	+	0	+	11	0	0	0	0
III	+	+	0	9	0	0	1	11
IV	+	+	+	13	0	13	7	54

into the passively sensitized mice failed to cause any deaths, the percentage of mice having tumor hemorrhage was the same as obtained with the actively sensitized animals. Tumors were transplanted into 6-week-old mice and 1 week later 0.5 ml. of mouse anti-BSA serum was injected intravenously. Twenty-four hours later 2.5 mg. of antigen (BSA) in 0.5 ml. of pyrogen-free saline was injected intraperitoneally. All of the sensitized animals showed shock reactions but none died. Seven of 13 animals showed a severe hemorrhagic reaction in the tumors.

The Effect of Cortisone on the Hemorrhagic Activity of the Factor From *E. coli*

The studies with cortisone were carried out in a manner similar to that described by Nelson *et al.* (*7*), who obtained an inhibiting effect on anaphylactic shock in mice. The *E. coli* material and method of testing for tumor hemorrhagic effect were the same as described by Ikawa *et al.* (*4*). Six-week-old mice were inoculated with tumors and 1 week later given 1 injection of 2.5 mg. of cortisone acetate (Upjohn). Twenty hours later, 10 μg. of THF was injected intra-abdominally. The data obtained from several experiments have been summarized in table 3. There was no statistical difference between the number of tumor hemorrhages in the cortisone-treated group and the control groups which received no THF. The protection induced by cortisone was quite significant and suggested that the THF activity might, in part at least, be due to an immune mechanism. It was also noted that the cortisone-treated mice did not exhibit the severe shock reaction of ruffled fur and prostration as did the tumor mice.

TABLE 3.—*Summary of the effect of cortisone on the activity of tumor hemorrhagic factor (THF) from* E. coli

Cortisone	THF	Number of mice	Mice showing hemorrhages in tumors (*number*)	Mice showing hemorrhages in tumors (*percent*)
+	+	41	11	27
0	+	35	25	71
+	0	29	5	17
0	0	25	6	24

Cortisone has many effects on physiological reactions, so that its role in the inhibition of the hemorrhage-producing activity of THF may not be the result of interference with antigen-antibody reaction. However, as a result of the studies of Farr and his associates (*2*), there seems to be little doubt but that immune mechanisms play some part in the pyrogenic activity of materials such as THF and hence it is highly probable that the hemorrhagic activity of THF also involves an immune mechanism. It is significant that the injection of THF into *normal* guinea pigs produced anaphylactic-like reactions and also gave typical Schultz-Dale reactions. Gross reactions in animals and isolated smooth muscle strips showed a relative specific refractoriness to a second exposure of antigen, indicating that the THF behaved as a typical antigen and that the animals were specifically sensitized. The present assumption is that sensitization probably arises from normal adsorption of *E. coli* or homologous antigens from other bacteria through the intestine.

Summary

The foregoing studies support the earlier findings of Barrett (*5*) which showed that anaphylaxis could induce hemorrhage in transplanted mouse

tumors. They also suggest that a specific immune mechanism may be involved in the activity of the tumor hemorrhagic factor from *E. coli*. The role of hypersensitivity in the THF reaction is also suggested by the inhibitory effect of cortisone.

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A Quantitative Comparison of the Antileukemic Effectiveness of Two Folic Acid Antagonists in Mice[1]

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In studies of the chemotherapy of neoplasia, increasing numbers of drugs have been demonstrated to possess tumor-inhibitory properties. This has made desirable the development of methods which will permit quantitative comparison of the relative effectiveness of such drugs.

A method has been described which provides a quantitative expression of the specificity of action of a treatment in terms of its effect on both tumor and host. It also permits a comparison of the antineoplastic specificity of action of differing treatments (*1*). The method has been employed to date in studies of the influence of the following factors on the antileukemic specificity of action of a drug in mice: *a*) the time of treatment following tumor implantation (*2*); *b*) the schedule of treatment (*2*); *c*) the administration of a metabolite with an antagonist, and the time of administration of the metabolite relative to the antagonist (*1*, *3*); and *d*) the age of the mice (*4*). It was observed that all these factors may influence the anti-tumor specificity of action of a drug.

The method has general applicability but as yet has not been employed to compare the relative antineoplastic effectiveness of different drugs. In the current investigation a quantitative comparison is made of the relative antileukemic effectiveness in mice of two well-known folic acid analogues, aminopterin and amethopterin, employing several treatment schedules. These two drugs were chosen since there has been considerable biological and clinical interest in the comparative antineoplastic effectiveness of the folic acid analogues (*5–10*).

Methods

The experimental animals were DBA/2J and CDBA[3] hybrid male mice, 10 to 13 weeks old. The tumor employed was the transplantable lymphoid leukemia L1210 carried in DBA/2J male mice. The experimental and statistical methods have been previously described (*1*). The animals were inoculated subcutaneously in the right thigh with 0.1 ml. of a uniform

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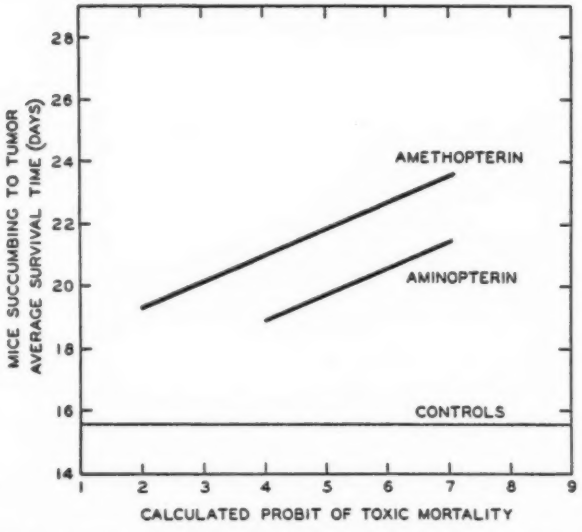
[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] (BALB/cAN × DBA/2J)F$_1$.

saline suspension of tumor cells. Amethopterin and aminopterin[4] were dissolved in 2-percent sodium bicarbonate. The drugs were administered subcutaneously in the axillary region in the constant volume of 0.01 ml. per gram. In each experiment, both the animals and the order of injection of the drugs were randomized. When more than one treatment was employed, the order of injection was randomized at each injection interval. The dosages employed and schedules of treatment are listed with the individual experiments.

Results

The results of three experiments are summarized in tables 1 through 3 and the corresponding text-figures 1 through 3. The first two experiments are essentially identical, the second being performed to confirm the results of the first. On four schedules of treatment the antileukemic specificity of action of amethopterin was greater than that observed for aminopterin. At equal probit of drug dose mortality, amethopterin was more effective than aminopterin in increasing the survival time of the leukemic mice when the drugs were compared (*a*) on single injection 3 days following tumor implantation (tables 1 and 2; text-figs. 1 and 2); (*b*) on multiple treatment



TEXT-FIGURE 1.—Effect of administration of aminopterin and of amethopterin on the survival time—toxic mortality relationship in leukemic mice. Inoculum 99,000 cells per mouse. Drug treatment 3 days following tumor inoculation (see table 1). Least-squares relationships between time to death from tumor for mice surviving aminopterin or amethopterin toxicity and the calculated probit of toxic mortality for the drugs were fitted. Separate common slope lines are shown for each treatment group. $P<0.025$ for the differences in the levels of the fitted lines. Control line is based on the average tumor survival time for 26 mice receiving no antagonist.

[4] Amethopterin and aminopterin were kindly provided by the Calco Chemical Division of the American Cyanamid Company, Bound Brook, N. J.

TABLE 1.—*Comparison of the antileukemic effectiveness of aminopterin and amethopterin**

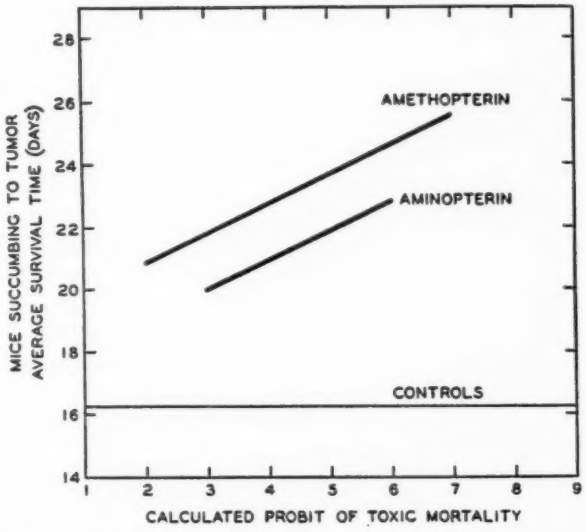
Drug deaths shown are among 13 treated animals; P=calculated probit of toxic mortality†; S. T./N=average survival time in days for mice surviving drug toxicity but succumbing to tumor/number of mice on which this average is based. Single treatment 3 days following tumor implantation.

Aminopterin dose (*mg./kg.*)	Drug deaths	P	S. T./N	Amethopterin dose (*mg./kg.*)	Drug deaths	P	S. T./N
1. 78	7	4. 98	19. 8/6	45. 6	0	2. 86	20. 8/13
2. 67	7	5. 32	19. 5/6	68. 4	2	3. 67	19. 6/11
4. 00	10	5. 66	20. 7/3	102	4	4. 49	20. 6/9
6. 00	12	6. 01	20/1	154	7	5. 30	24. 0/6
9. 00	11	6. 35	22/2	231	11	6. 11	21. 5/2
13. 5	12	6. 69	21/1	346	13	6. 92	.....
20. 2	13	7. 04	.....	519	13	7. 73	.....
30. 4	13	7. 38	.....	778	13	8. 54	.....

Controls: 26 mice in various control groups (2-percent $NaHCO_3$; untreated) all died of tumor between the 14th and 19th day following tumor inoculation; average=15.6 days.

*DBA/2J male mice; inoculum 99,000 cells per mouse.

†Calculated probits obtained from the fitted maximum likelihood dose-response curves. Individual probit slopes were fitted for the two treatment groups using one cycle of the Cornfield-Mantel procedure (*12*).



TEXT-FIGURE 2.—Effect of administration of aminopterin and of amethopterin on the survival time—toxic mortality relationship in leukemic mice. Inoculum 54,000 cells per mouse. Drug treatment 3 days following tumor inoculation (see table 2). Least-squares relationships between time to death from tumor for mice surviving aminopterin or amethopterin toxicity and the calculated probit of toxic mortality for the drugs were fitted. Separate common slope lines are shown for each treatment group. $P<0.001$ for the differences in the levels of the fitted lines. Control line is based on the average tumor survival time for 24 mice receiving no antagonist.

TABLE 2.—*Comparison of the antileukemic effectiveness of aminopterin and amethopterin**

Drug deaths shown are among 12 treated animals; P=calculated probit of toxic mortality†; S. T./N=average survival time in days for mice surviving drug toxicity but succumbing to tumor/number of mice on which this average is based. Single treatment 3 days following tumor implantation.

Aminopterin dose (*mg./kg.*)	Drug deaths	P	S. T./N	Amethopterin dose (*mg./kg.*)	Drug deaths	P	S. T./N
0. 78	0	3. 07	19. 9/12	30. 2	1	2. 69	22. 6/11
1. 18	2	3. 72	20. 5/10	45. 6	0	3. 38	21. 8/12
1. 78	3	4. 37	20. 6/8‡	68. 4	2	4. 07	22. 2/10
2. 67	8	5. 02	21. 5/4	102	4	4. 75	23. 3/7‡
4. 00	5	5. 66	24. 6/5§	154	7	5. 44	24. 7/3§
6. 00	12	6. 31	.....	231	11	6. 13	23. 0/1
9. 00	12	6. 96	.....	346	12	6. 81	.....
13. 5	12	7. 61	.....	519	12	7. 50	.....
20. 2	12	8. 26					

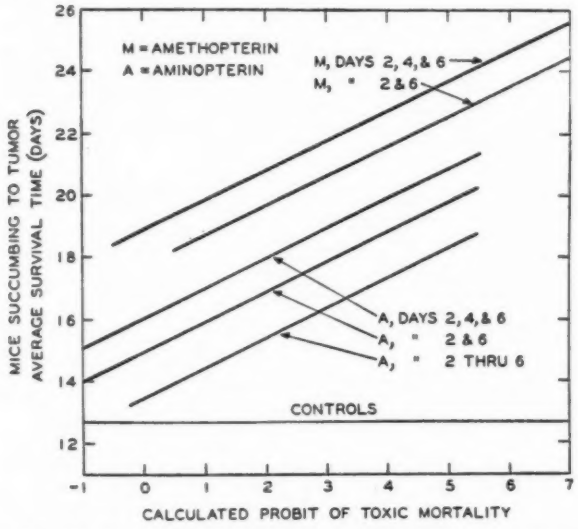
Controls: 24 mice in various control groups (2-percent $NaHCO_3$; untreated) all died of tumor between the 14th and 19th day following tumor inoculation; average=16.3 days.

*DBA/2J male mice; inoculum 54,000 cells per mouse.

†Calculated probits obtained from the fitted maximum likelihood dose-response curve. Individual probit slopes were fitted for the two treatment groups using one cycle of the Cornfield-Mantel procedure (*15*).

‡One survivor at 90 days following tumor implantation.

§Two survivors at 90 days following tumor implantation.



TEXT-FIGURE 3.—Effect of multiple treatment schedules of aminopterin and amethopterin on the survival time—toxic mortality relationship in leukemic mice (see table 3). Inoculum 550,000 cells per mouse. The days of treatment indicated are the days following tumor inoculation. Least-square relationships between time to death from tumor for mice surviving aminopterin or amethopterin toxicity and the calculated probit of toxic mortality for the drugs were fitted. Separate common slope lines are shown for each treatment group. $P<0.001$ for the differences in the levels of the fitted lines. Control line is based on the average tumor survival time for 97 mice receiving no antagonist.

TABLE 3.—*Comparison of the antileukemic effectiveness of aminopterin and amethopterin employing several multiple treatment schedules**

Drug deaths shown are among 10 treated animals; P=calculated probit of toxic mortality†; S. T./N=average survival time in days for mice surviving drug toxicity but succumbing to tumor/number of mice on which this average is based.

Days of Drug Administration (Day of Tumor Inoculation=Day 0)

Total dose (*mg./kg.*)	Days 2 and 6			Days 2, 4, and 6			Days 2 through 6		
	Drug deaths	P	S. T./N	Drug deaths	P	S. T./N	Drug deaths	P	S. T./N
Aminopterin									
0. 28	.....	.....	.....	.....	.....	.....	0	−0. 23	13. 8/10
0. 47	.....	.....	.....	0	−1. 26	14. 2/10	0	1. 17	15. 4/10
0. 78	0	−0. 92	13. 7/10	0	0. 18	17. 2/10	0	2. 57	15. 9/10
1. 30	0	0. 34	15. 0/10	0	1. 62	17. 9/10	1	3. 97	16. 3/9
2. 16	0	1. 61	16. 8/10	1	3. 05	18. 3/9	8	5. 37	18. 0/2
3. 60	0	2. 87	18. 3/10	1	4. 49	20. 6/9	9	6. 77	17. 0/1
6. 00	3	4. 13	19. 0/7	9	5. 93	21. 0/1	10	8. 17	.....
10. 0	5	5. 40	19. 6/5	10	7. 37	.....	10	9. 57	.....
16. 7	10	6. 66	.....	.....	.....	.....	.....	.....	.....
Amethopterin									
6. 00	.....	.....	.....	.....	.....	.....	0	(‡)	16. 5/10
10. 0	.....	.....	.....	0	−0. 30	18. 2/10	0	.....	17. 2/10
16. 7	0	0. 70	17. 9/10	0	1. 16	20. 4/10	0	.....	18. 1/10
27. 8	0	1. 76	18. 2/10	0	2. 61	21. 6/10	2	.....	19. 0/7§
46. 3	0	2. 83	20. 5/10	1	4. 06	22. 7/9	10	.....	.....
77. 2	1	3. 89	22. 9/9	9	5. 52	25. 0/1	10	.....	.....
129	5	4. 95	23. 8/5	9	6. 97	24. 0/1	10	.....	.....
214	10	6. 02	.....	10	8. 42	.....	10	.....	.....
357	9	7. 08	23. 0/1	10	9. 87	.....	10	.....	.....
595	10	8. 15	.....	.....	.....	.....	.....	.....	.....

Controls: 97 mice in various control groups (2-percent $NaHCO_3$; untreated) all died of tumor between the 11th and the 16th day following tumor implantation; average=12.7 days.

*CDBA hybrid male mice; inoculum 550,000 cells per mouse.

†Calculated probits obtained from the fitted maximum likelihood dose-response curves. Individual probit slopes were fitted for the various treatment groups using one cycle of the Cornfield-Mantel procedure (*12*).

‡Data do not permit calculation of probits.

§One mouse apparently tumor-free at 90 days.

schedule days 2, 4, and 6 (table 3, text-fig. 3); (*c*) multiple treatment schedule days 2 and 6 (table 3, text-fig. 3); (*d*) although the survival time-probit curves are not presented[5] for treatment on days 2 through 6, the data (table 3) suggest that here too, for equal cost in drug dose mortality, amethopterin was more effective than aminopterin in prolonging the survival time of the leukemic mice.

For both analogues, daily multiple treatments appeared to be the least effective of the multiple treatments employed (table 3, text-fig. 3).

Discussion

The quantitative procedure employing the survival time-probit line, developed to evaluate the chemotherapeutic effectiveness of individual drugs and to gain information concerning the drug-host-tumor relation-

[5] See footnote, table 3.

ship in the whole animal (*1–3*), can be applied to compare the antileukemic specificity of action of two different drugs.

This comparison, as carried out in our laboratory and using the mouse as a test animal, has shown that amethopterin is more effective than aminopterin in the treatment of leukemia L1210. Whether the observed difference in effectiveness of the two drugs indicates some difference in mechanism of action is not clear. One of the alternate possibilities is that differences in contained impurities (*11*) may account for the greater effectiveness of amethopterin.

These experiments illustrate that it is not necessary for a drug to show increased potency at equal dose with respect to the tumor in order to produce a greater specificity of antileukemic action. The specificity of antileukemic action is governed by the effect of the drug on the host as well as on the tumor.

An increase in antileukemic specificity of action of a drug or a drug treatment involves a relative increase in the inhibitory effect of the drug with respect to the tumor as compared with the toxicity to the host (*3–5*). The greater specificity of antileukemic action of amethopterin as compared with aminopterin comes about in the following manner:

a) For equal dose, amethopterin is far less toxic for the host than is aminopterin. The range of effective doses and the LD_{50} for amethopterin is on the order of 15 to 70 times that for aminopterin (tables 1–3; table 4, column 1).

b) Also, amethopterin is far less toxic for the tumor than is aminopterin. Considerably higher doses of amethopterin are required to obtain the same survival time yielded by a given dose of aminopterin (tables 1–3; table 4, column 3).

TABLE 4.—*Comparison of aminopterin and amethopterin with respect to tumor and host*

Experiment and treatment	Comparison at equal host mortality		Comparison at equal survival time: aminopterin LD_{50} survival time*	
	1	2	3	4
	LD_{50} (*mg./kg.*)	Survival time at LD_{50} (*days*)	Dosage (*mg./kg.*)	Host mortality (*percent*)
I.† Aminopterin, day 3	1. 83	19. 8	1. 83	50
Amethopterin, day 3	133	21. 9	38	0. 6
II.‡ Aminopterin, day 3	2. 65	21. 9	2. 65	50
Amethopterin, day 3	119	23. 7	38	2. 7
III.§ Aminopterin, days 2 and 6	8. 5	19. 8	8. 5	50
Amethopterin, days 2 and 6	131	22. 6	33	0. 2
Aminopterin, days 2, 4, and 6	4. 3	20. 9	4. 3	50
Amethopterin, days 2, 4, and 6	64	23. 7	23	0. 2

*Dosage shown is that required to obtain the same survival time yielded at the aminopterin LD_{50} in the particular experiment. Host mortality shown is that resulting from this required dosage.

†See table 1 and text-figure 1. Average survival time for controls is 15.6 days.

‡See table 2 and text-figure 2. Average survival time for controls is 16.3 days.

§See table 3 and text-figure 3. Average survival time for controls is 12.7 days.

c) However, though amethopterin is less toxic than aminopterin for both the host and the tumor, the reduction in toxicity is more pronounced for the host. For example, a much smaller dose of amethopterin than its host LD_{50} is necessary to produce the same survival time as does the aminopterin LD_{50} (table 4, columns 1 and 3). Thus amethopterin is able to achieve the same anti-tumor effect as aminopterin at a considerably lower cost in toxicity to the host (table 4, column 4). This, in turn, is reflected in longer survival times for equal toxicity (*i.e.*, greater antileukemic specificity) for amethopterin than for aminopterin (table 4, column 2; text-figs. 1–3).

Summary

Quantitative methods, previously developed and applied to the study of host-tumor relationships as affected by a specific drug, have been utilized to compare the antileukemic effectiveness of aminopterin and amethopterin. As employed by us in the mouse, amethopterin was found to be more effective than aminopterin in the treatment of leukemia L1210. It produced greater survival times of leukemic mice than did aminopterin for the same cost in drug lethality to the mice.

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On the Etiology of Esophageal Carcinoma[1, 2]

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The question as to whether human cancer is determined by heredity or caused by exogenous factors has been debated since the beginning of the 18th century, but it was not until the 20th century that a number of case histories stimulated major investigations of its familial occurrence. This question was often complicated by the tendency to look upon cancer as an undifferentiated entity, without regard to the organ affected. A long time elapsed before it was realized that owing to the increase in the average length of life the risk of developing most varieties of cancer had become so great that nearly all families with a few elderly members counted one or more cases of cancer, without this fact being interpretable as an increased prevalence of cancer in the family concerned. In many recent studies, however, the categories of relatives have been accurately defined, the sex ratio and age distribution calculated, and the number of cancer cases compared with the prevalence of cancer in satisfactory control series.

Several varieties of cancer have recently been the subject of statistical-genealogical analysis in an endeavor to elucidate the significance of genetic factors.

According to Jacobsen (*1*) the heredity of breast cancer appears to be dominant with failing manifestation. He maintained that he had demonstrated a general predisposition to endogenous cancer as well as a localization factor. Subsequent investigations also speak for the existence of a gene that is specific to breast cancer (*2*, *3*).

Videbæk (*4*) studied the families of 209 patients with leukemia. Irregular dominance was considered the most likely mode of inheritance. The increased prevalence of cancer among the relatives was interpreted to indicate a nonspecific cancer gene.

Investigating uterine cancer, Brøbeck (*5*) concluded that cancer of the uterine body must be governed by a general cancer gene as well as by an inherited localization factor. Cervical cancer, on the other hand, seemed to be of exogenous origin.

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In Murphy's opinion (*6*), the hereditary factors influencing the development of uterine cancer are specific and do not predispose to cancer of other organs to any extent worth mentioning. Murphy, however, made no distinction between cancer localized to the body and the cervix.

Videbæk and Mosbech (*7*) analyzed the possible causal factors in gastric carcinoma. The occurrence of cancer among the relatives of 302 patients with gastric carcinoma was studied. The findings were compared with the prevalence in control data from healthy persons whose corresponding relatives were of the same sex and age distribution, and who had been questioned just as thoroughly. Cancer of the stomach proved to be more common among the relatives of the diseased persons, whereas cancer of other sites appeared to be of the same distribution in both groups. The risk for the relatives of the patients acquiring gastric carcinoma proved to be four times that for the control groups.

As these findings could not be explained by external factors, such as occupation, eating, smoking or drinking habits, or socioeconomic circumstances, it was concluded that the excess mortality from gastric carcinoma among the relatives of the patients must be genotypically determined. Cancer of other sites occurred about as frequently among the relatives of the patients as among those of the controls, so a general cancer gene could not be adduced for this type of carcinoma.

On the basis of these results, and in an attempt to shed some more light on the heredity of cancer, the authors studied another tumor of the alimentary tract—cancer of the esophagus.

Literature

In several respects cancer of the esophagus differs essentially from alimentary tumors of the lower tract. Histologically, it is a squamous-cell carcinoma. The disease is far more common in men than in women, and the age distribution shows that this disease often affects the very old.

Little is known about the etiology. Persons in poor socioeconomic circumstances have been reported to be those chiefly affected (*8*). In a French study, a large alcohol consumption was found in 95 percent of 110 patients with cancer of the esophagus (*9*), and a recent British study showed excess mortality from esophageal cancer among brewers, barmen, and waiters (*10*). Otherwise, exact data about these aspects are scarce.

The drinking of copious amounts of excessively hot tea has been mentioned as a predisposing factor for Russian immigrants in the United States of America (*8*).

In the presence of corrosive strictures in the esophagus, the risk of esophageal cancer is increased (*11*). Syphilis has been mentioned as a predisposing factor (*12*), but this matter has not yet been clarified.

Sideropenic anemia with dysphagia and glossitis—the Plummer-Vinson syndrome, which affects only females—is not infrequently followed by high-seated esophageal carcinoma (*13*).

The typical Plummer-Vinson syndrome is rare in Denmark, and recent studies indicate that its etiologic role in carcinoma of the upper alimentary tract is unimportant in this country (*14*).

No attempt seems to have been made heretofore to discover whether there is a hereditary factor in esophageal cancer.

Material

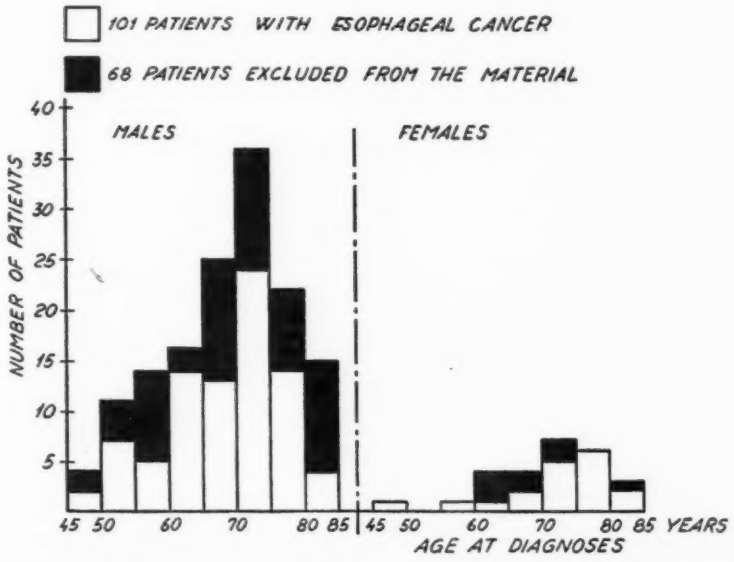
The material was derived from the Radium Centre, Copenhagen, and comprised patients with proved esophageal carcinoma admitted during the period from 1947 to 1951. In addition, it included 48 patients (43 males and 5 females) collected from the Radium Centre in 1942 by E. Mogensen for the purposes of a family study. Mogensen did not complete his study, but kindly placed the material at the present authors' disposal. From the total material of 169 patients, 68 had to be discarded for the reasons shown in table 1. The remaining 101 patients consisted of 83 males and 18 females.

TABLE 1.—*Grounds for the exclusion of 68 patients*

	Number
Uncertain diagnosis	14
Illegitimacy	6
Deficient family data	31
Family refused to collaborate	17

The sex ratio ♂/♀ of about 5/1 accords well with previous publications (*10, 15*).

Text-figure 1 gives the age distribution at the time of diagnosis. It will be seen that the distribution remained unchanged after the exclusion



TEXT-FIGURE 1.—Age distribution of patients at time of diagnosis.

of the 68 patients. The average age at onset was 67.9 years for men and 71.6 years for women.

The diagnostic criteria given in table 2 show that the diagnosis was verified histologically in the great majority of cases. In a few cases, autopsy was performed without microscopic examination of the tumor, and in 10 percent the diagnosis was based exclusively on characteristic X-ray appearances and the subsequent course of the disease.

TABLE 2.—*Diagnostic criteria in the 101 cases of esophageal carcinoma*

	Males	Females	Total
Histologic diagnosis	72	13	85
Autopsy without histologic examination	5	1	6
X-ray examination only	6	4	10
Total	83	18	101

Table 3 shows the site of the esophageal tumors. More than half were situated in the middle third, less than one third were localized close to the cardia, and about 10 percent were high-seated.

TABLE 3.—*Site of tumor in 101 cases of esophageal carcinoma*

Site of tumor in esophagus	Males	Females	Total
Upper third	8	2	10
Middle third	48	8	56
Lower third	23	5	28
Unknown	4	3	7
Total	83	18	101

Method

At the time of the study all the patients with esophageal cancer had died. Their next-of-kin were contacted and questioned, partly to supplement the sparse data of the case records regarding diet, alcohol consumption, etc., and partly to obtain details about the patients' relatives (names, diseases, causes of death, and other data).

This part of the study, including the verification of the collected particulars, was performed in the same detailed way that has been described in previous papers by the authors (*4*, *7*, *16*), using the proband method and including the groups of parents, siblings, and children in the investigation.

Results

a) *Alcohol.*—The patients' alcohol consumption was analyzed. Among 84 men whose alcohol consumption was known, 54 (65%) had for a long time daily consumed liquor to an extent sufficient for their next-of-kin to

characterize them as alcoholics. Eighteen (21%) of the 84 men were in exposed occupations (waiters, 10; brewery workers, 4; representatives, 3; wine and spirit merchants, 1). One of the 18 women was described as an alcohol addict, and another was employed at a brewery.

b) *Syphilis.*—Empirically, it is difficult to glean reliable data regarding syphilis by questioning patients. In order to elucidate the possible etiologic role of syphilis, records of 118 patients with esophageal carcinoma—98 males and 20 females—in the syphilis register in the State Serum Institute in Copenhagen were searched. This register, which was started in 1920, comprises all syphilitics in Denmark who have given positive Wassermann reactions. The identification of the patients on this roster is only by initials, sex, date and year of birth, and name of their physician, so it does not preclude some inaccuracy. However, all doubtful cases were ruled out, leaving only those syphilitics whose identity was beyond question. The numbers found must therefore be considered minimum values.

This search revealed that 10 of the 98 men (10.2%) had a definite history of syphilis. None of the 20 women was on the register. Thus a total of 8.5 percent of the 118 patients had a history of syphilis.

For purposes of comparison, it is worth mentioning that in a similar study of 708 patients with gastric carcinoma the authors found a definite history of syphilis in 6.0 percent of the male and in 3.6 percent of the female patients. The slightly higher prevalence of syphilis among the patients with esophageal carcinoma is not significant, however.

In 2,000 patients consecutively admitted to a Danish medical department, 10 percent were syphilitics (*17*).

Accordingly, the prevalence of syphilis found among the patients with esophageal carcinoma does not afford any evidence of an etiologic relationship between syphilis and esophageal carcinoma.

c) *Anemia.*—An attempt was made to trace a history of Plummer-Vinson syndrome (hypochromic anemia with glossitis and dysphagia) in the 18 women of the series, but with a negative result. Perhaps this explains why cancer affecting the uppermost part of the esophagus—postcricoid cancer, which other workers have found particularly frequently in women of the younger age groups (*13*)—occurred in only 2 of the 18 patients in the present series.

d) *Heredity.*—As already mentioned, previous authors have demonstrated the etiologic significance of hereditary factors in many varieties of cancer that have proved to be of familial occurrence.

In the present study, the incidence of cancer among the relatives of the patients with esophageal cancer was compared with the incidence in a control series. As many of the patients with esophageal cancer had died several years before the investigation was performed, it was necessary to operate with a corrected age distribution for the probands and to use a similarly distributed control series.

The patient material comprised 877 relatives (423 males and 454 females) averaging 8 to 9 for each of the 101 probands with esophageal carcinoma.

There were 80 cases of cancer (9.1%) among the relatives. In 5 instances (6.3%) the tumor was situated in the esophagus. Table 4 gives the distribution of the cancer cases in the various categories of relatives. In 76 instances, the diagnoses of cancer had been verified in the hospital or by death certificate, whereas in 4 cases the diagnoses were based exclusively on data regarding the course of the disease given by the family.

TABLE 4.—*Occurrence of total cancer and esophageal cancer in the various categories of relatives in the patient series (P) and control series (C)*

	Relatives (*number*)		Cases of total cancer (*absolute number and percent*)		Cases of esophageal cancer (*number*)	
	P	C	P	C	P	C
Fathers	99	276	11 (11%)	28 (10%)	1	7
Mothers	100	278	20 (2%)	40 (14%)	0	1
Brothers	219	645	21 (10%)	45 (7%)	3	2
Sisters	241	654	22 (9%)	51 (8%)	0	2
Sons	105	371	2 (2%)	5 (1%)	1	1
Daughters	113	348	4 (4%)	7 (2%)	0	0
Total	877	2,572	80 (9.1%)	176 (6.8%)	5	13

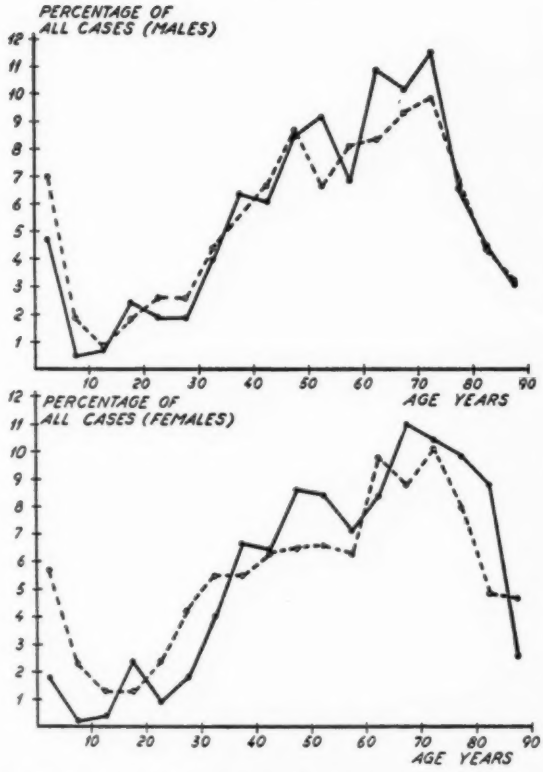
The control series comprised 341 healthy persons (209 males and 132 females) whose relatives were studied with regard to diseases and causes of death, there being an average of 8 relatives for each proband. This group was identical with the control series used by the authors in three previous studies (*4*, *7*, *16*) to which the reader is referred for further details.

The group of control probands was derived from the originally larger control material by excluding at random a number of persons from the younger age groups so that the percentile age distribution became identical with that of the patient group.

The relatives of the control group totaled 2,572 persons—1,292 males and 1,280 females. The percentile age distribution of relatives in the patient and control groups is given for males and females in text-figure 2. It will be seen that the control series includes somewhat too many relatives under 30 years of age. Since cancer is rare in these age groups, this slight disproportion is of no importance. Statistical calculation shows the age distribution for persons over 30 years of age to be the same in both groups. The two groups are therefore comparable with regard to sex and age.

Among the 2,572 relatives in the control series there were 176 cases of cancer (6.8%). Thirteen (7.5%) of these patients had cancer of the esophagus.

In other words, the study showed the prevalence of esophageal cancer among the relatives of patients suffering from this disease to be the same



TEXT-FIGURE 2.—Percentile age distribution of relatives of patients—solid lines; and of controls—dotted lines.

as that in an adequate control series. Familial occurrence of esophageal carcinoma could not be substantiated.

There was a slightly higher prevalence of total cancer among the relatives of the patient group (9.1%) than among those of the control group (6.8%), but the difference is not significant ($t=1.22$, P is between 20 and 25%).

Cancer of the esophagus thus corresponds to cancer of the uterine cervix, but pathogenetically it appears to differ from cancer of the breast, cancer of the body of the uterus, and cancer of the stomach, which are of familial occurrence, and must therefore be considered as partially determined by endogenous factors.

Discussion and Conclusion

The present investigation did not reveal any accumulation of cancer of the esophagus or of other sites among the relatives of patients with esophageal cancer. The study thus failed to show any influences of hereditary factors.

Provided that the resistance to cancer is the same in both sexes, the marked male preponderance is compatible with the supposition that the disease is exogenous in nature and that men are more exposed to the carcinogenic action.

The large number of old patients in the series indicates that the cancer-accelerating factor has a long latent period or that it has to be present for many years before carcinoma develops.

As regards dietetic factors of possible etiologic significance, it is reasonable to suggest excessive consumption of alcohol. Other studies point to there being a relationship between the abuse of alcohol and esophageal cancer (*9*).

The large proportion of alcohol addicts in the present series thus confirms previous observations and points to alcohol possessing decisive etiologic significance for this type of cancer, at least for men.

An analysis of which forms of alcohol are particularly operative in causing the malignant changes in the squamous epithelium of the esophagus was impracticable because of the difficulty of procuring sufficiently accurate data regarding the amount and nature of the patients' liquor consumption.

A retrograde analysis of eating habits, complicated by the inaccurate data obtained by this technique, failed to afford conclusive evidence that particularly hot food should be an etiologic factor.

No definite relationship could be demonstrated between bad teeth and esophageal cancer.

No correlation was found between syphilis and esophageal cancer in the present series. Indeed, it is difficult to conceive the mechanism of such a relationship.

Analysis of the relationship between sideropenic anemia and esophageal cancer gave a negative result. This is not surprising, however, considering the small number of female patients in the present series and the relatively rare occurrence of sideropenia in Denmark.

Summary

The etiology of esophageal carcinoma was studied on the basis of a series comprising 83 males and 18 females with tumors the diagnosis of which was incontestable.

Abuse of alcohol is an important associated factor in the etiology of esophageal carcinoma, at least for men, 65 percent of the patients having been alcohol addicts and 21 percent having had alcohol-exposed occupations.

Syphilis was not recorded more often than in a mixed series from a hospital department. This disease is therefore hardly of pathogenetic significance. The Plummer-Vinson syndrome did not occur among the patients.

The study failed to show any influences of hereditary factors, there being the same incidence of esophageal, as well as total, cancer in 877 relatives of patients with cancer of the esophagus and in 2,572 corresponding relatives of healthy persons.

Cancer of the esophagus must therefore be considered to be essentially due to exogenous factors.

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Studies of the Mechanism of Action of Urethan in Initiating Pulmonary Adenomas in Mice. I. The Indirect Nature of Its Oncogenic Influence[1,2]

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Work in this laboratory has been primarily directed toward finding the mechanism of action of certain oncogenic agents. A necessary requisite to such an investigation is the determination of whether the individual agent is acting directly upon the tissues as such or through intermediary substances. Several types of information from previous investigations suggest that urethan may act directly on the lung; namely, the rapidity of the catabolism and excretion of urethan (*1*), its rapid fixation in cells (*2*), the direct nature of its nuclear poisoning effects (*3*), the fact that lung tissue does not develop tumors when implanted in animals as little as 24 hours after injection of the recipients with urethan (*4*), and the fact that as a result of painting the skin with both urethan and croton oil (*5*) skin tumors are increased in number over the number elicited on other mice with croton oil alone. The evidence reported herein, on the contrary, shows that urethan acts through an intermediary substance in producing pulmonary adenomas in mice.

Materials and Methods

Mice of the BALB/c (*6*) and A (*7*) strains, originally obtained from Dr. Peyton Rous of the Rockefeller Institute and bred since in this laboratory, were used.

The fetal lung tissue used in these experiments was procured and implanted by the general methods outlined by Rous and Smith (*8*). The methods of exposure of the lung tissue to the oncogen are, in general, variations of those which have proved useful in *in vitro* studies of the carcinogenic influences of both nitrogen mustard (*9*) and ultraviolet light (*10*) inducing pulmonary adenomas of the sort produced by urethan injected into intact mice (*11*). As these differ to a considerable extent from experiment to experiment they will be individually described. In experiments where minced lung tissue was exposed *in vitro* to the oncogen the fragments of tissue were less than 0.2 mm. across. After exposure all

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tissues were implanted in the posterior thigh muscles of unexposed mice of the same strain, providing the tissue under test. Following a period varying from several weeks to several months the implants were excised and examined microscopically in serial section for the presence of tumors.

Experiments and Results

Experiments 1 through 9.—Lung tissue from fetuses late in gestation was hashed in isotonic Ringer's solution and exposed to urethan for different periods and at the concentrations as indicated in table 1. The fragments were then rinsed in Ringer's solution, and implanted in unexposed animals of the same strain. Each implanted mouse carried two implants, one of tissue exposed to urethan in one leg and another of unexposed lung tissue from the same source in the opposite leg.

The animals used in experiments 2, 3, 4, 5, 7, and 8 were of the BALB/c strain. Those of experiments 1, 6, and 9 were of the A strain. The concentration of urethan used was obtained by diluting isotonic urethan with Ringer's solution to the concentration desired. Except in experiments 7 and 8, finely hashed lung tissue was exposed to the drug. In experiments 7 and 8 the hearts of the fetuses were tied off and the lungs injected by intratracheal cannulation and, after the period of exposure indicated, the lungs were hashed and rinsed in Ringer's solution. After a period varying from 2 to 12 months the implanted mice were killed and their implants examined for tumors.

No adenomas or tumors of other types were found in any of the 124 implants examined (see table 1).

As no direct oncogenic effect of urethan was apparent in experiments 1 through 9, it seemed possible that the duration of exposure of the lung tissue to the oncogenic agent might be a critical factor in explaining the failure of tumors to appear. The experiment that follows shows that the times of exposure of the lung tissue to urethan in these first experiments would have been sufficient if the oncogen had been urethan.

It has been reported by Kline (*12*) that urethan crosses the placenta to the fetus and results in the initiation of pulmonary adenomas in the newborn animals delivered by cesarean section as little as 5 minutes after injection of the mother with urethan. A modification of this procedure was used in the following experiment but with the lungs of the fetuses excised and implanted in unexposed animals after varied short periods following injections of the pregnant females with urethan.

Experiment 10.—Nine pregnant BALB/c mice of an estimated 18 to 20 days' gestation were injected intraperitoneally with 1.5 mg. of urethan per gram mouse. The urethan was given as a 5-percent solution in distilled water. One and a half hours later the lungs of the fetuses of 3 of these pregnant females were excised and hashed in Ringer's solution and implanted in the thigh muscles of 10 unexposed young adults. This same procedure was carried out on the other two groups of 3 mice each, 3 and 5 hours, respectively, after injection of the pregnant females with urethan. Eleven weeks later, the implants were excised and examined

TABLE 1.—*Results of exposure of lung tissue of mice to urethan* in vitro *and subsequently implanted intramuscularly into mice of the strain of origin*

Experiment	Fetuses yielding tissue (*number*)	Strain of mice	State of tissue when exposed	Urethan concentration (*percent*)	Conditions of exposure	Mice implanted (*number*)	Examination time after implantation (*months*)	Condition of implants	Adenomas found (*number*)
1	6	A	Hashings...	0.5	In Ringer's for 1 hour.....	8	2	Good.....	0
2	11	BALB/c	Hashings...	.5	In Ringer's for 2 hours.....	7	6	Excellent.....	0
3	9	BALB/c	Hashings...	.5	In Ringer's plus glucose and ⅓ rabbit serum for 1.5 hours.	5	3	Excellent.....	0
4	10	BALB/c	Hashings...	.5	In Ringer's plus glucose and ⅓ rabbit serum for 2 hours.	5	5	Excellent.....	0
5	11	BALB/c	Hashings...	.5	In Ringer's plus glucose and ⅓ mouse serum for 2 hours.	5	4	Excellent.....	0
6	10	A	Hashings...	.5	In Ringer's plus ⅓ mouse serum for 24 hours.	5	2	Only bronchial epithelium.	0
7	10	BALB/c	*In situ*.....	.8	In Ringer's plus glucose for 2–3 hours.	13	4–12	Excellent.....	0
8	13	BALB/c	*In situ*.....	1.5	In Ringer's plus glucose for 2–3 hours.	10	6–12	Excellent.....	0
9	10	A	Hashings...	5.0	In Ringer's for ½ hour.....	4	3	Only bronchial epithelium.	0

in serial section for the presence of tumors. Seven mice from the group of 30 implanted were discarded because of weight loss or intercurrent disease.

Four adenomas were found in the implants of the lung tissue exposed *in utero:* one in the tissue exposed for 1½ hours, one in that exposed for 3 hours, and two in that exposed for 5 hours. The periods of exposure used in experiments 1 through 9 were sufficient for the agent to have acted in its oncogenic capacity if urethan had been the oncogen.

From experiments with nitrogen mustard, however, it is known that the initiation of these tumors is highly conditioned by the state of the cells at the time of exposure (*9*). It seemed possible that the direct *in vivo* exposure of lung tissue to urethan might exert an oncogenic influence despite the negative result of the *in vitro* studies. As the injection of functioning lung seemed likely to result in drowning, the injection of implanted fetal lung was resorted to. It has been shown that adenomas develop in implants of lung tissue carried by mice given urethan (*4*).

Experiment 11.—The lungs from 15 fetuses (15 to 21 mm. crown-rump) were obtained from 3 pregnant BALB/c mice, hashed in Ringer's solution, and implanted bilaterally in the thigh muscles of 10 young adult animals of the same strain. Three weeks later when the implanted tissue was distinctly palpable, 0.2 cc. of an isotonic mixture of urethan (2.8%) in Ringer's solution was injected into one or the other leg implants of the individual hosts. Ringer's alone was injected in the opposite implant. Two and a half months later the animals were killed and the implants examined for adenomas.

Again no direct influence of the drug, as such, on the lung tissue was manifest. Three tumors appeared—two in implants of the Ringer's injected controls and one in a urethan-injected implant. As the hosts carrying these implants had each received an estimated ¼ mg. of urethan per gram mouse and since this amount in a single injection had been previously shown to produce adenomas in the lungs of mice (*13*), it is not remarkable that these few tumors appeared in the implants.

The above experiments made it seem likely that there was an intermediary substance in the serum of mice injected with urethan which was exerting the oncogenic influence. The difficulty in getting a sufficient volume of serum from mice under the proper conditions necessitated the use of larger animals. Rats, guinea pigs, and rabbits were resorted to. Following injection with urethan, rats developed pulmonary adenomas morphologically similar to those which develop in mice (*14*). Guinea pigs developed adenomas following injection with methylcholanthrene (*15*), but they have thus far proved resistant to the oncogenic influence of urethan (*16*). Rabbits have never been reported to develop pulmonary adenomas either spontaneously or under experimental conditions. It was determined to find out whether an oncogenic influence would be exerted on implants of mouse lung tissue in rats, guinea pigs, and rabbits. Newborn recipients were used, as it is well known that the newborn will better tolerate the implantation of foreign tissue than the mature animal and also will permit growth of these tissues for a short time.

Experiment 12.—The lungs from 17 fetal strain BALB/c mice, 22 to 24 mm. crown-rump, and from 2 pregnant females were hashed in Ringer's solution and implanted in the thigh muscles of 3 newborn rabbits, 3 newborn guinea pigs, and 3 weanling mice of the BALB/c strain. Twenty-four hours later two animals of each species group were given 1.5 mg. of urethan per gram of body weight intraperitoneally. One animal of each group received no injection. Twenty-four hours after injection [3] the implanted mouse lung tissue was excised, separated from extraneous tissue, hashed, rinsed in Ringer's solution and implanted in the thigh muscles of weanling mice. Three mice were implanted with the lung implants of each of the three groups of animals used; the lung tissue which was exposed to the influence of urethan injection in the temporary host was implanted opposite that tissue that had been carried by the unexposed animal of the same species. After an interval of 2½ months the mice were killed and the implants were examined in serial section for the presence of tumors.

Four adenomas were found in the implants exposed in rabbits, two of these tumors in one implant. One adenoma was found in the implants of tissue exposed in weanling mice. No adenomas were found in the implants of the lung tissue exposed to urethan in guinea pigs. None of the implants carried by intermediary hosts not given urethan developed adenomas.

The following experiment is similar to experiment 12, except that rats and mice were used as the intermediary hosts in which the mouse lung tissue was exposed to the oncogenic effect of injection of urethan.

Experiment 13.—The lungs from the fetuses of 2 pregnant BALB/c mice late in gestation were used. One of these animals yielded 11 fetuses, 20 to 22 mm. crown-rump (an estimated 18–19 days' gestation), and the other 5 fetuses, 13 to 15 mm. crown-rump (an estimated 14–15 days' gestation). The lungs were pooled and hashed in Ringer's solution as above. The suspension of lung in Ringer's solution was implanted in 0.2 cc. amounts into the thigh muscles of 6 rats and 3 weanling mice. After 24 hours, 3 of the rats were given urethan and all of the mice were given 1.5 mg. of urethan per gram of body weight intraperitoneally. After 24 hours the implanted lung was excised, hashed, and reimplanted as in experiment 12. Three mice were implanted with the tissue exposed in rats, the exposed lung tissue being implanted opposite that carried by the rats not given urethan. Three mice were implanted with the lung tissue exposed in the weanling mice. After an interval of 2½ months, the animals were killed and the implants examined for tumors as usual.

One adenoma was found in each of the implants of tissue which had been exposed to the oncogenic influences of urethan injection in the newborn rats. None were found in the implanted tissue carried by the intermediary host rats not injected with urethan. An adenoma was found in two of the three implants exposed in the intermediary weanling mice injected with the drug.

[3] At the time of excision of the implants, the injected mice had fully recovered from the anesthetic effects of urethan, regaining consciousness about 3 hours after injection. Both the rabbits and guinea pigs were still conscious at this time.

It is evident that mouse lung tissue exposed to the oncogenic influence of urethan injection in either rats, rabbits, or other mice develops adenomas. Too few guinea pigs were used to judge whether the lack of responsiveness of the tissue is significant.

These results indicate that the serum of either rats or rabbits could be used in direct tests for the presence of an oncogenic intermediate. In the experiments that follow, a comparative test of oncogenicity was made between rabbit serum to which urethan was added *in vitro* and serum from the same animal taken several hours after urethan injection.

Experiment 14.—A New Zealand Red rabbit, weighing 2,550 grams, was bled aseptically from the heart into a 10-cc. syringe containing 0.2 cc. of 1:100 heparin solution. The blood was immediately centrifuged and the plasma separated. The rabbit was then given 1 mg. of urethan per gram of body weight intraperitoneally. Five hours later the rabbit was again bled from the heart, 50 cc. of blood being drawn into a syringe containing 1 cc. of 1:100 heparin solution, and the plasma was separated by centrifugation.

Three pregnant BALB/c mice yielded 7, 6, and 10 fetuses measuring, respectively, 15, 17, and 22 mm. crown-rump. The lungs were excised and pooled, hashed in Ringer's solution and divided into two parts. One part was added to 2 cc. of the plasma taken before urethan injection and to which urethan was added to a concentration of 1 percent.[4]

The remaining half of the lung tissue was added to 2 cc. of the rabbit plasma taken 5½ hours after injection of the animal with urethan. The tissue was exposed to these sera *in vitro* with intermittent agitation at room temperature for 3 hours, then rinsed in Ringer's solution and implanted in the opposite thighs of 20 young adult BALB/c mice. After an interval of 2½ months the animals were killed and the implants examined for the occurrence of tumors.

Four of the implants of tissue exposed to the rabbit serum taken after injection of the animal with urethan had one adenoma each. No adenomas were found in the implants of lung exposed to serum from the same rabbit to which urethan had been added *in vitro*. In the following experiment the same result was obtained.

Experiment 15.—This experiment was identical to experiment 14 except that 12 mouse fetuses from two pregnant females were used. There were 9 animals in one litter and 3 in the other. These varied from 19 to 24 mm. crown-rump and were an estimated 18 to 20 days' gestation. The lung tissues exposed to the serum taken after injection, but with urethan added *in vitro* to a concentration of 1 percent, were implanted in opposite thighs of 10 young adult BALB/c mice. After an interval of 3 months the animals were killed and the implants examined for tumors.

Five of the implants of tissue exposed to the rabbit serum taken after injection of the animal with urethan were positive for adenomas; one of these implants had two tumors. None of the implants of tissue exposed

[4] This concentration of urethan has been reported to be the level maximally present in the serum of rabbits given 1.5 mg. of urethan per gram of body weight intraperitoneally (*17*).

to the rabbit serum to which urethan was added *in vitro* developed adenomas.

Experiments 14 and 15 indicate that the oncogenic influence of urethan injection in the production of these adenomas is consequent to the elicitation of an oncogenic intermediate.

Discussion

The results of these experiments make it plain that the action of urethan to initiate pulmonary adenomas is through an intermediary substance or substances. They cast no light upon the question of whether the urethan molecule is transformed into the intermediate or whether its action is to elicit a chemically unrelated substance. In either case it is evident that the active intermediate is produced by both the rat and rabbit as well as the mouse. As morphologically identical adenomas appear in the lungs of both rats and mice following exposure of these animals to urethan, there is no reason to believe that the oncogenic agent responsible for this neoplastic change in these two species differs in character. The rabbit has never been reported to develop these tumors spontaneously or after treatment with a wide variety of carcinogenic agents. In unpublished work done in this laboratory, 30 rabbits did not develop pulmonary adenomas in a period of 6 months following four injections of 1 mg. of urethan per gram of body weight given at weekly intervals. It appears probable that rabbits are constitutionally incapable of developing this individual neoplasm yet are able to produce the oncogenic intermediate following their injection with urethan. In the case of the guinea pig the number of animals tested in experiment 12 is too small to warrant a conclusion, but it seems pertinent to point out that this species has been shown to develop pulmonary adenomas following injection of methylcholanthrene (*15*) but not after urethan (*16*). Future experiments may show whether these findings are the result of an inability of this species to make the oncogenic intermediate.

The recent report of Salaman and Roe (*5*) concerning the use of urethan as an initiator of skin tumors in the mouse appears at first sight contradictory to the above results with lung tumors, for they painted the skin of the animals directly with urethan. However, the authors point out that at the dosage of urethan used in their experiments enough was absorbed to produce a narcotizing effect on the painted animals. As pulmonary adenomas have been produced by painting the skin of mice with urethan (*18*) there is no reason to believe that an oncogenic intermediate might not as well be required for the initiation of skin tumors with this agent, the intermediate after its elicitation traveling to the skin and exerting an oncogenic effect. However, it is conceivable that the epithelial cells of the skin might be able to produce the oncogenic intermediate, while those of the lung require a systemic transformation or production of the oncogen.

Considering the similarities in the initiation of these pulmonary adenomas with nitrogen mustard (*9*) or ultraviolet irradiation (*10*) and the

induction of back-mutation in *Neurospora* with these same agents (*19*), it is noteworthy that urethan as such has had no demonstrable mutagenic effect in these organisms (*20, 21*). A mutagenic effect has, however, been demonstrated following injection of *Drosophila* with urethan (*22*). Whether this disparity in mutagenicity is related to the formation of an intermediary substance in *Drosophila*, such as has proved the case with the oncogenic activity of urethan exposure in mice, has yet to be shown.

Summary

Urethan is shown to have no oncogenic influence when applied directly to pulmonary epithelium. Tumors were observed in mouse lung tissue exposed to the oncogenic effects of urethan injection in rats, rabbits, and mice, and transplanted to unexposed mice for manifestation of the tumors.

The presence of an oncogenic intermediary substance in the serum of rabbits given urethan is demonstrated by *in vitro* exposure of lung tissue from fetal mice to such serum. Simultaneous exposure of lung tissue from the same mice to serum from the same rabbits, but taken prior to urethan injection and with urethan added *in vitro*, yielded no tumors.

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Induction of Skin Tumors in Strain DBA Mice Following Intermittent Treatment With 9,10-Dimethyl-1,2-Benzanthracene and Croton Oil[1,2]

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It has been demonstrated in the mouse that application to the skin of one subminimal dose of a carcinogen produces a lasting and possibly irreversible alteration (*1*). Visible skin tumors may be induced if repeated applications of croton oil, considered to be noncarcinogenic, are subsequently administered to the area painted with carcinogen (*2–4*). This "promoting" action of croton oil is reversible. If, following repeated applications, treatment with croton oil is interrupted for 9 or more weeks and then resumed, the latent period of tumorigenesis appears to be determined by the paintings subsequent to, but not prior to, the interruption (*5*, *6*). Recently, it was reported from this laboratory (*7*), that strain DBA mice exposed to one dose of methylcholanthrene and repeated doses of croton oil bore fewer skin tumors, and these after a longer period of time, when the croton oil was applied at intervals of 2 weeks as compared to twice weekly. Inasmuch as the decreased "promoting" action in the previous investigation (*7*) was associated with an increased interval between applications of croton oil, it was suggested that the development of visible tumors might be retarded or prevented by extension of this interval to 1 month. In addition to studying the "promoting" influence of monthly applications of croton oil following one subminimal dose of the carcinogen, 9,10-dimethyl-1,2-benzanthracene, it was decided to investigate whether substitution of additional monthly applications of the same dose of carcinogen for the croton oil would be more effective in skin tumorigenesis. After exposure to one subminimal dose of 9,10-dimethyl-1,2-benzanthracene, some of the mice were given additional doses equivalent to the first one but at intervals of 2 and 3 months to determine whether visible tumors could be induced despite these prolonged rest periods.

Materials and Methods

A total of 223 strain DBA/2 Jax male mice 6 to 7 weeks of age were clipped free of hair in the region of the nape of the neck and 0.05 ml. of a

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1.5-percent solution of 9,10-dimethyl-1,2-benzanthracene [4] in acetone was applied once by pipette to each mouse. The mice were housed in metal cages in air-conditioned quarters and had access to an unlimited supply of Purina laboratory chow and tap water. One month following skin painting (at this time none of the mice had a visible tumor) 143 of the animals were divided into several groups and 0.05 ml. of the following solutions was applied monthly to the area exposed initially to DMBA: group I—no further treatment; group II—a 2.5-percent solution of croton oil [5] in olive oil for duration of experiment; group III—2 additional paintings with 1.5-percent DMBA; group IV—4 additional paintings with DMBA; group V—6 additional paintings with DMBA. Eighty other mice which also had been painted once with DMBA, as described previously, were divided into two groups and treated as follows: group VI—at intervals of 2 months, applications of DMBA to the same site and at the same dosage as that employed initially or treatment continuing for the duration of the experiment; group VII—same treatment as group VI except for extension of interval between paintings to 3 months.

The mice were examined periodically for tumors, and the size, position, and number of growths per animal noted. Tumors which were below 1 mm. in size and which did not persist at least 2 weeks were not included in this study. At autopsy, tumors and surrounding skin were excised, fixed in Tellyesniczky's fluid, sectioned at 5 to 8 micra, and stained with hematoxylin and eosin. Tumor diagnoses were confirmed following microscopic examination.

Results and Discussion

No visible tumors were induced among the mice exposed to one application of 1.5-percent DMBA in acetone (group I). Ten of these mice were observed for 175 days and the remaining 10 for 298 days. Among the mice painted once with DMBA followed by repeated monthly paintings with croton oil (group II), 2 out of 21 were observed with tumors, 1 papilloma per mouse. The results obtained in group II demonstrate little or no "promoting" influence for croton oil under the conditions employed. A number of the mice received as many as 11 applications of the oil but bore no visible tumors. It appeared that some or all of the enhancing effects induced with each application of croton oil disappeared before the next dose was applied. In a previous study from this laboratory it was reported that although croton oil exerted a "promoting" influence when administered once every 2 weeks, this influence was significantly less than that obtained following application twice weekly (7). These results together with those observed in the present investigation indicate that more applications with shorter intervals between applications are required for the development of visible tumors with croton oil. This would suggest that some of the croton oil or a metabolite thereof persists during the brief interval producing a more or less continuous stimulus.

[4] Hereafter referred to as DMBA.

[5] Obtained from Fisher Scientific Company, New York, N. Y.

TABLE 1.—*Influence of intermittent skin painting with 9,10-dimethyl-1,2-benzanthracene and croton oil in strain DBA male mice*

Group	Treatment*	Total DMBA applied (*mg.*)	Effective total† (*number*)	Mice with tumors (*number and %*)	Tumor multiplicity‡ (*number*)	Latent period§ (*days*)	Observation period (*average days*)
I	D. 1 ×	0. 75	20	0	———	———	237
II	D. 1 ×; c. o. 1 ×/ mo.	0. 75	21	2/9. 5	1. 0	———	310
III	D. 1 ×/mo. for 3 mos.	2. 25	38	34/89. 5	4. 2	80	153
IV	D. 1 ×/mo. for 5 mos.	3. 75	17	16/94. 1	8. 1	88	157
V	D. 1 ×/mo. for 7 mos.	5. 25	32	30/93. 8	9. 3	72	155
VI	D. 1 ×/2 mos. . .	3. 00	39	26/66. 7	2. 8	162	200
VII	D. 1 ×/3 mos. . .	2. 25	37	14/37. 8	1. 6	>218	218

*D. = DMBA; c. o. = croton oil.
†Survivors at time first tumor was observed.
‡Average number of tumors per tumor-bearer.
§Time when 50% of mice bore tumors measured from start of experiment.

A high incidence of tumors was obtained for the mice exposed to three monthly applications of DMBA (group III, table 1). However, tumors also were observed following two such applications. Thus, 11 out of 38 mice in group III, 6 out of 17 in group IV, and 12 out of 32 in group V bore 1 or more tumors by the 59th day. These data demonstrate that following one subminimal application of DMBA another equivalent application is considerably more effective in eliciting visible tumors than a series of monthly applications of croton oil (group II). Fifty percent of the mice in groups III, IV, and V bore skin tumors after approximately the same latent period—72–88 days (column 7, table 1). Most of these tumor-bearers had been exposed to equivalent doses of DMBA by this time (*i.e.*, three doses), since the fourth dose was applied to groups IV and V 90 days from the start of the experiment. Although more tumors per mouse were observed in groups IV and V than in group III (column 6, table 1), about the same number of mice bore tumors in each of these groups (column 5, table 1). All surviving mice in groups III, IV, and V had visible skin tumors by the end of the experiment. However, the total tumor incidence fell below 100 percent for each of these groups because some of the mice included in the effective totals died without tumors before the experiment was ended.

Among the mice which received one application of DMBA every 2 months (group VI), 26 out of 39 were susceptible and bore a total of 73 tumors. Although the mice received a total of 4 applications of DMBA, seven animals were observed with tumors after only two doses had been applied, *i.e.*, 98 days from the start of the experiment. In group VII, where DMBA was applied once every 3 months, 14 out of 37 mice were observed with tumors, an average of 1.6 tumors per tumor-bearer. Thus tumor multiplicity for the latter group was approximately half that

obtained in group VI (column 6, table 1). Four of the mice in group VII bore visible skin tumors by the 176th day, or before application of the third dose of DMBA. It is apparent from these data that exposure to two subminimal doses of a carcinogen is sufficient to induce skin tumors in the mouse even when such doses are separated by a period as long as 3 months. The fact that tumors were produced among the mice exposed to DMBA monthly (groups III, IV, and V), once every 2 months (group VI), and even once every 3 months (group VII) is substantial evidence in favor of a cumulative influence for DMBA in skin tumorigenesis. A similar effect was not demonstrable with monthly applications of croton oil (group II). It is unlikely that any of the original carcinogen remained after 3 months when the second dose was applied (group VII). It is recognized that nonfluorescent metabolites may be produced following application of DMBA and that one or more of these may have remained after the parent compound could no longer be detected by fluorescence.

It has been observed in other experiments in this laboratory with strain DBA mice painted once with a subminimal amount of a carcinogen, that the tumors subsequently produced with croton oil are almost all benign (*6–8*). In the present experiment, squamous-cell carcinomas as well as benign papillomas were produced in response to the three, five, or seven monthly paintings with DMBA (groups III, IV, and V). Four of the mice in group III, three in group IV, and four in group V bore one carcinoma each during the course of the experiment. Although Salaman and Roe (*9*) reported that malignant skin tumors also are obtainable in mice exposed to one subminimal dose of a carcinogen followed by treatment once weekly with the "promotor," croton oil, these appeared only when the observation period was extended to one year or longer (*9*). In the studies reported herein, limited monthly applications of a low dose of DMBA appear to be more effective in the development of skin carcinomas in the mouse than one application of DMBA followed by weekly applications of croton oil.

Summary

Skin tumorigenesis was investigated in strain DBA mice following one exposure to a 1.5-percent solution of 9,10-dimethyl-1,2-benzanthracene (DMBA) in acetone. Some of the mice received no further treatment, while in others the painted site was exposed monthly to a 2.5-percent solution of croton oil in olive oil or 1.5-percent DMBA in acetone, or to the same dose of carcinogen applied once every 2 or 3 months. The mice painted monthly with DMBA were distributed among three groups which received a total of three, five, or seven applications of the carcinogen. A tumor incidence of 90 to 94 percent and tumor multiplicities of 4.2, 8.1, and 9.3 percent, respectively, were obtained for these three groups during an average observation period of 153 to 157 days. Two out of 21 mice (9.5 percent) exposed to croton oil monthly bore one papilloma each during an observation period of 310 days. A tumor incidence of 67 percent was observed for the group of mice exposed to DMBA once every 2 months. When the interval between exposures was extended to 3

months, 38 percent of the mice bore tumors. Tumor multiplicities for the 2- and 3-month groups were 2.8 and 1.6, respectively, while the corresponding observation periods were 200 and 218 days. Although tumors were not observed among the mice exposed to one dose of DMBA alone, some of the mice from each group given additional doses developed skin tumors after two applications of the carcinogen.

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Effect of X Irradiation on the Growth of the Ehrlich Ascites Tumor[1,2]

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In previous communications (*1*, *2*), methods were described for the quantitative measurement of ascites tumor growth in mice. After intraperitoneal inoculation of known numbers of cells, the increase in the total number of free tumor cells in the peritoneal fluid was determined and the growth curves so obtained were analyzed in some detail.

In the present investigation an attempt was made to determine quantitatively the effect of X irradiation on the growth of the ascites tumor. Different doses of X rays were administered to ascites tumor cells before inoculation and the total number of free tumor cells in the peritoneal fluid was counted at various time intervals after inoculation. The findings were compared with the data obtained previously for the growth of the nonirradiated tumor. Studies were also made of the changes in the number of free nontumorous cells and the total volume of ascitic fluid. Particular attention was given to the effects of the presence of dead or lethally damaged tumor cells after irradiation.

Materials and Methods

Mice

Heterozygous albino mice, received from a commercial farm, were used. All experiments were carried out with either male or female mice 2 to 3 months of age and had a body weight of 20 to 22 grams. The animals were kept on a standard diet. Food and drinking water were available *ad libitum*.

Tumor

The Ehrlich ascites tumor was employed for all experiments. This tumor was produced in this laboratory from a solid Ehrlich carcinoma in 1948 (*3*) and, since then, has been maintained by serial ascitic transfers

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in hybrid mice (over 260 passages). The transfers were carried out routinely by injecting 0.1 to 0.2 ml. of the undiluted ascitic fluid intraperitoneally. The growth curves obtained with untreated cells used as controls in the present experiments are similar to those reported in 1953 (*1*).

Irradiation

Irradiation of the tumor cells was performed *in vitro* under nitrogen in an ice bath. For this purpose, a cylinder-shaped irradiation chamber of methacrylate polymer with an airtight lid was devised. It had a height of 15 mm. and a radius of 30 mm. The atmosphere was controlled through two glass capillary tubes which passed through the lid. N_2 gas was led through a gas washer filled with water before being used. The chamber, containing about 6 cc. of ascitic fluid, and immersed in ice water, was perfused with N_2 gas for 15 minutes before, and during, irradiation. The ascitic fluid was kept in constant motion by the stream of gas. X rays were generated at 145 kv. and 6 Ma. filtered by 1 mm. Al, HVL 0.14 mm. Cu. The top of the irradiation chamber was at a distance of 67 mm. from the X-ray tube. The dosage delivered to the cells was determined by means of ionization chambers. During the time when the irradiation was being carried out, control tumor cells were kept in a refrigerator at 4° C.

Procedure

Five to 7 days after the inoculation of 0.2 cc. of undiluted ascitic fluid, the ascites of 2 or 3 mice was collected and pooled. Part of the fluid was used for cell counting in a Buerker hemocytometer and for preparing fresh smears that were fixed and stained according to the Papanicolaou method (*4*). Another aliquot was exposed to a given dose of X rays. After irradiation, measured volumes of undiluted ascitic fluid, containing 18×10^6 tumor cells were injected intraperitoneally into each of 25 to 30 mice. The course of ascites tumor growth was then followed in the injected animals by sacrificing two mice daily and determining the ascites volume, and the total number of tumorous and nontumorous cells by a dye-dilution method as described previously (*2*).

Results

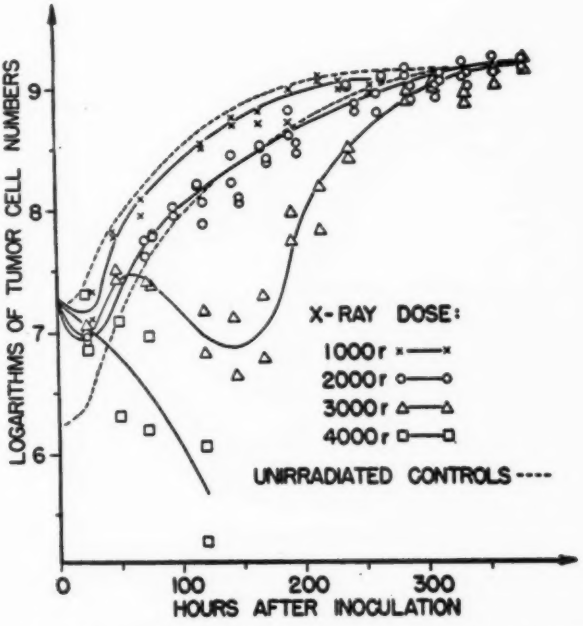
Number of Tumor Cells

In the first experiment of the present series X-ray doses of 400, 1,000, 2,000, 3,000, and 4,000 r were used and the total number of free tumor cells in the peritoneal fluid was studied as a function of time after the inoculation of 18×10^6 irradiated cells. All irradiations were carried out in a nitrogen atmosphere. The growth curve obtained after irradiation with 400 r did not differ from that established for the untreated tumor (*1*). Text-figure 1 shows the effect of the higher doses. Twenty-four hours after inoculation there was a decrease in the number of tumor cells in every case. This was not observed when the same number of unirradiated

tumor cells was inoculated. After 4,000 r the number of tumor cells continued to decrease and they finally disappeared from the peritoneal fluid. With doses between 1,000 and 3,000 r, in all cases, the initial decrease was followed by an increase in cell number, reaching values higher than the number of inoculated cells within 72 hours. Cells irradiated with 1,000 r showed a slightly slower increase in number than untreated cells. With a dose of 2,000 r, growth was considerably slowed down and the resulting growth curve was similar to that obtained after the inoculation of 1.8×10^6 nonirradiated cells (*1*). The growth curve after irradiation with 3,000 r showed, in addition to the initial decrease and recovery, a secondary decrease, beginning at about 60 hours and leading to a minimum value 150 hours after inoculation. This was followed by a new increase in cell number.

For each X-ray dose, except 4,000 r, the total number of tumor cells reached a plateau at about 10^9. This figure is similar to the number of cells in the final population of the nonirradiated tumor.

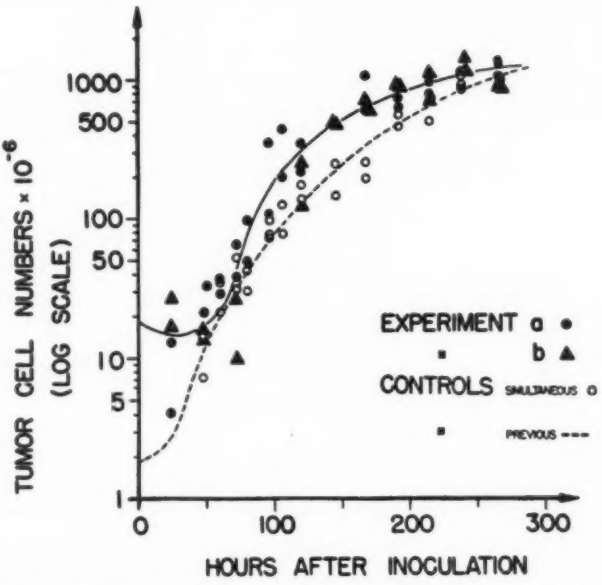
After irradiation with 2,000 r, the growth curve of 18×10^6 irradiated cells showed a course similar to that obtained when 1.8×10^6 non-



TEXT-FIGURE 1.—Changes in the total number of irradiated and nonirradiated free Ehrlich ascites tumor cells in the entire peritoneal fluid of the mouse. The continuous lines represent irradiated cells. In each case 18×10^6 tumor cells were inoculated intraperitoneally after irradiation with different X-ray doses. Each point was obtained by using a different mouse. The interrupted lines represent the increase in cell number after inoculation of 18×10^6 (*upper curve*) and 1.8×10^6 (*lower curve*) nonirradiated Ehrlich ascites cells.

irradiated cells were inoculated. This effect could be interpreted in a number of ways. One possibility was that nine tenths of the cell population used for inoculation was lethally damaged by irradiation and the growth observed might be attributed to the remaining one tenth. To test this possibility, the following experiment was performed:

Nonirradiated tumor cells, 1.8×10^6, were mixed with 16×10^6 cells originating from the same pool but having received a dose of 5,000 r. Cells exposed to these doses of X rays do not produce tumors when inoculated into the peritoneal cavity. A group of mice simultaneously injected



TEXT-FIGURE 2.—Changes in the total number of free Ehrlich ascites tumor cells in the entire peritoneal fluid of the mouse. The continuous line shows the changes in cell number after 1.8×10^6 nonirradiated cells were mixed and inoculated together with 16×10^6 cells previously irradiated with 5,000 r. Data are results of two separate experiments. Each point was obtained by using a different mouse. The growth of nonirradiated controls is represented by open circles from data obtained simultaneously as well as by an interrupted line based on previous experiments with over 200 animals.

with 1.8×10^6 nonirradiated tumor cells served as controls. Results of two separate experiments of this kind (*a* and *b*) are shown in text-figure 2. The growth curve obtained for the controls was similar to those repeatedly observed in the past after inoculation of 1.8×10^6 Ehrlich cells. It will be seen that the admixture of irradiated cells gave rise to a growth curve differing in shape from that obtained with 1.8×10^6 nonirradiated cells alone. Thus, after 96 hours the total number of tumor cells in the mixed population reached values higher than those of the controls, the mean counts being 100 percent greater at 144 and 192 hours. Finally,

both populations reached the same maximum size and the differences disappeared.

In order to investigate further this apparent growth stimulation produced by radiation-damaged cells, another experiment was performed. In this case the proportion of living to lethally damaged cells was made smaller.

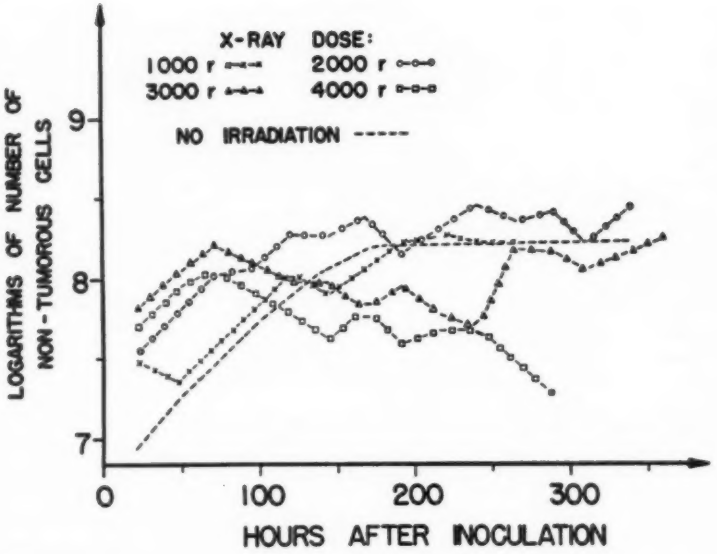
One hundred nonirradiated cells suspended in Krebs-Ringer phosphate (*p*H 7.3) were mixed with 18×10^6 cells previously irradiated with 5,000 r and injected in each of eight mice (group A). Another group of 8 mice (group B) received 100 nonirradiated cells, while a third group (group C) was inoculated with 18×10^6 cells irradiated with 5,000 r. All animals of group B succumbed with ascites tumors within 1 month after inoculation. In group A only one animal developed an ascites tumor and no tumors were obtained in group C during an observation period of 3 months. After this time, members of groups A and C were challenged by injection of 13×10^6 untreated Ehrlich cells. Two months later, all animals were still alive. The maximum survival time of mice receiving 1×10^6 tumor cells is usually about 3 weeks (*5*). The inhibition of tumor development initiated by the irradiated cells thus appears to extend over a period of at least 5 months.

Number of Nontumorous Cells

About 15 percent of the cell population in the well-developed Ehrlich ascites tumor consists of nontumorous cells (lymphocytes, granulocytes, histiocytes and mesothelial cells). Thus, in an inoculation of 18×10^6 tumor cells, about 3.2×10^6 nontumorous cells are simultaneously injected. The normal, untreated mouse with a body weight of about 20 grams has an average of 4.8×10^6 cells in the peritoneal fluid. Provided that no inflammatory reaction follows inoculation, and if the inoculated cells are not destroyed during the first 24 hours after inoculation, the total number of nontumorous cells on the day after inoculation should thus average about 8 million. A value of this magnitude could indeed be found in the nonirradiated tumor (*1*). After the inoculation of irradiated cells, the number of nontumorous cells was, however, much higher, ranging between 20×10^6 and 80×10^6 on the day after inoculation. Larger X-ray doses were followed by a more intense inflammatory reaction, as illustrated by text-figure 3. During the further course of the growth, the number of nontumorous cells paralleled the number of tumor cells, in a manner similar to that shown for nonirradiated cells.

It was suggested previously (*1*) that the number of free nontumorous cells in the Ehrlich ascites tumor was a function of the tumor-cell number. The following equation was found to fit closely the data obtained with nonirradiated cells:

$$b = \frac{100}{1 + k + \frac{e_o}{a}} \qquad [1]$$

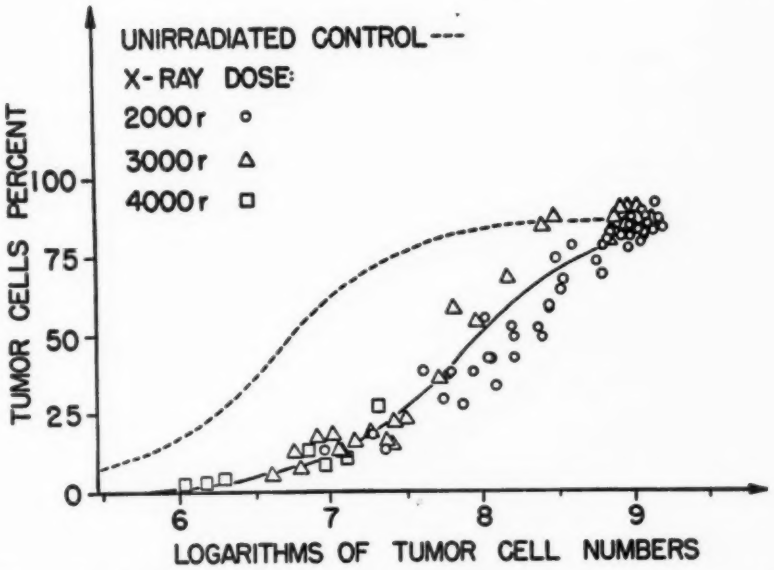


TEXT-FIGURE 3.—Changes in the total number of nontumorous cells in the entire peritoneal fluid during the growth of the irradiated Ehrlich ascites tumor. The data were obtained from the same experiment as shown in text-figure 1. The interrupted line represents the increase in the number of nontumorous cells after inoculation of 18×10^6 nonirradiated Ehrlich ascites cells.

where b=percentage of tumor cells in the fluid, a=total number of tumor cells, k=a constant and e_o=average number of free nontumorous cells in the peritoneal fluid of an untreated mouse of the same weight. When the data of the present irradiation experiments were plotted in the same way (b against log a) and compared to the previous curve, a shift to the right was observed (text-fig. 4). This may be explained as being due to an increase in the basic number of nontumorous cells (represented by e_o) as a result of the primary inflammatory reaction described above. The increase is different for the different X-ray doses. An average value of 85×10^6 fits the experimental findings as shown in text-figure 4. This observation indicates that the number of nontumorous cells in an irradiated population increases in essentially the same way as described previously for untreated cells except for a primary inflammatory reaction that follows the injection of irradiated cells.

Amount of Peritoneal Fluid

The amount of peritoneal fluid that is formed after the inoculation of the tumor cells was calculated from the experimentally determined values for ascites volume (V) by subtracting 0.12 ml. (the average volume of fluid in untreated mice of the same weight, V_o). In the untreated Ehrlich ascites tumor, V-V_o is a linear function of tumor-cell number during the major part of the growth period (*1*). This is also approximated by the



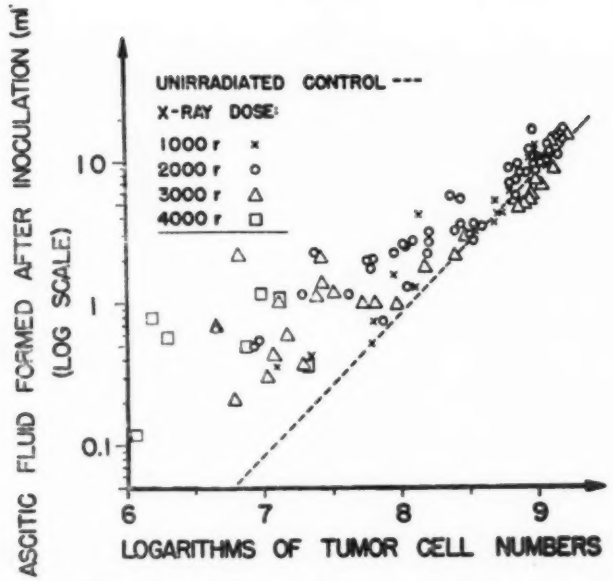
TEXT-FIGURE 4.—Relationship between the percentage of tumor cells, (b) and the logarithms of their total number (log a). The data have been obtained at different times after the inoculation of 18×10^6 Ehrlich ascites tumor cells irradiated with different X-ray doses. (Same experiments as shown in text-fig. 1.) The interrupted line shows the relationship observed with the nonirradiated tumor. The continuous line was obtained from equation [1], where e_o (the basal number of nontumorous cells in the peritoneal cavity of the mouse) was given a value of 85×10^6.

latter part of the growth curve obtained with irradiated cells (text-fig. 5). However, the relationship does not hold for the earlier part, where the value V-V_o per tumor cell is increased.

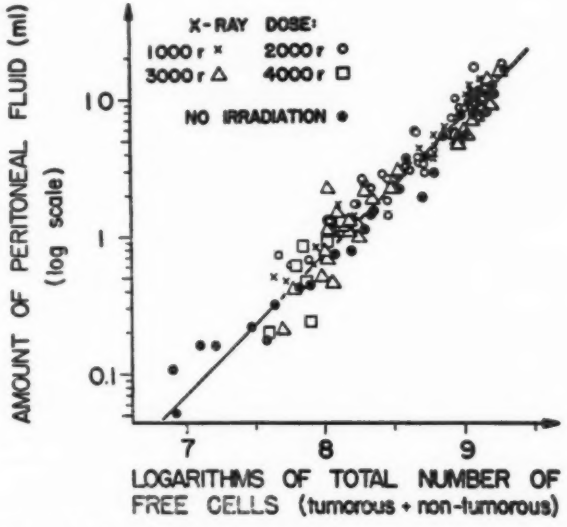
The results further show that a direct proportionality exists between the total number of cells and the volume of fluid in the peritoneal cavity (text-fig. 6). This constancy of concentration ($123–143 \times 10^6$ cells per ml.) is maintained throughout the growth period. It is uninfluenced by shifts in the cellular composition of the ascites fluid. [In the early phases after inoculation, inflammatory cells represented nearly 100% of the population, while at the end of the growth period about 85% were tumorous cells (text-fig. 4)]. It is observed with irradiated as well as nonirradiated cells and with all doses of X rays examined.

Discussion

The experimental findings show that depending on their quantitative proportion, tumor cells lethally damaged by X rays can exert either an inhibiting or an enhancing effect on the growth of viable cells with which they are mixed. This is at variance with the conclusions of Henshaw (*6*), who inoculated subcutaneously varying proportions of untreated and irradiation-killed cells from Sarcoma 37 into DBA mice. Since he found no differences in survival time between their various groups, he con-



TEXT-FIGURE 5.—Relationship between the number of free-tumor cells in the peritoneal cavity and the volume of ascitic fluid formed after intraperitoneal inoculation of irradiated tumor cells. The ordinates represent the total amount of ascites after subtraction of the average volume of peritoneal fluid in normal mice (0.12 ml., ref. *1*). The interrupted line shows the relationship observed with the nonirradiated tumor.



TEXT-FIGURE 6.—Relationship between the total number of free cells (tumorous + nontumorous) in the entire peritoneal fluid and the volume of ascites. The line corresponds to an average cell concentration of 133×10^6 cells per ml.

cluded that the cells killed by X ray had no influence on the growth of the tumor. However, minor differences in growth might not be detectable by survival time measurements. In the present work, differences in growth rate which were observed early, leveled out toward the end of the growth period.

In the case of a large number of nonirradiated cells mixed with a large number of radiation-killed cells, growth was enhanced. This suggests that dead or dying cells might release substances that stimulate tumor growth, an effect which might be observable only when the number of viable tumor cells is large. An alternate possibility is that part of the tumor population survived exposure to 5,000 r. H. M. Patt has suggested[4] that these surviving cells might have been incapable of growth in the face of the severe inflammatory reaction evoked by the cells that were killed by X rays. This situation, however, might not have occurred if a large number of nonirradiated cells had been present. The irradiated cells that were still alive might then have had a better chance to grow and their numbers would have been added to those of the whole growing population. This would have produced an apparent enhancement in the growth.

In the case of a small number of nonirradiated cells mixed with a large number of radiation-killed cells, growth was inhibited. The mice survived for 3 months without signs of tumor development and a second challenge with nonirradiated tumor cells in high doses did not result in the development of progressively growing tumors. The most plausible interpretation of this finding is that the radiation-damaged cells induced a state of resistance against Ehrlich ascites tumor cells. This type of "immunization" of the host by damaged tumor cells has been well established for similar host-tumor systems and has been shown to be due to isoantigenic differences that exist between a tumor and its host when these are genetically unrelated. Goldfeder (*7*) has demonstrated the induction of a state of resistance similar to that observed in the present experiments in cases where irradiation-damaged tumors grew in foreign host genotypes.

Possibly related to the present findings are the results of Kaliss, Snell and other authors [reviewed by Snell (*8*)]. These workers obtained either decreased or increased susceptibility to homologous tumor transplants by treating mice with heat-killed or frozen-dried tumor tissues 1 to 3 weeks prior to implantation. They showed that the difference between resistance and enhancement was dependent upon dosage—small amounts of killed material giving resistance; large doses, enhancement.

The question of how many cells in an irradiated population are lethally damaged by different doses of X rays does not receive a simple numerical answer from growth-curve studies. While 18×10^6 cells irradiated with 2,000 r had a course of growth similar to that of 1.8×10^6 nonirradiated cells, the experiments with mixed populations show that this does not simply mean that lethal damage was inflicted upon nine tenths of the cell population. Furthermore, the total regression of tumor cells within a few days after irradiation with 4,000 r does not necessarily mean that all

[4] Personal communication.

Ehrlich cells were killed by this dose. Instead, a large number of damaged cells may have immunized the host against the survivors and this may have been responsible for the observed regression.

The depressing effect of X rays on the increase in the number of tumor cells that could be observed in every case after irradiation with doses of 1,000 r or more is thus probably the final result of a complex situation. In addition to mitotic inhibition, killing and lethal damage to cells, such phenomena as immunization of the host by dead or dying cells and stimulation of the survivors by products released from the disintegrating elements may all play a role.

Inflammatory Reaction

Irradiation of the Ehrlich ascites tumor produced a leukocytic reaction similar to that described by previous investigators in solid tumors (*9*, *10*). The inflammatory response was evident soon after the inoculation of the irradiated cells into the peritoneal cavity and its intensity was directly related to the X-ray dose. Since the irradiations were performed *in vitro*, this response can not be attributed to a direct effect of X rays on the host. It must result from some quality of the inoculated cells. The fact that a relation exists between the dose of X rays to which these cells were exposed and the intensity of the inflammatory reaction that followed their implantation suggests that products of the disintegrating elements are responsible for the effect. Klein and Forssberg (*11*) arrived at a similar conclusion from studies on *in vivo* irradiated Ehrlich ascites tumor.

Ascitic Volume

After the inoculation of irradiated Ehrlich cells into the peritoneal cavity of the mouse, fluid accumulated rapidly. In the early stages of growth, the volume of ascites was unusually high and not directly proportional to the number of tumor cells present. Later, the increase in peritoneal fluid volume was directly related to the total number of free tumor cells, a phenomenon already observed in the case of the nonirradiated Ehrlich tumor (*1*).

The early fluid accumulation was probably part of the initial inflammatory reaction associated with the presence of cells damaged by irradiation. The proportionality between ascites volume and tumor-cell number observed in the later stages may indicate that ascitic fluid is produced as a result of the metabolic activity of the neoplastic cells themselves, as proposed earlier (*2*).

Summary

The growth of *in vitro* irradiated Ehrlich ascites tumor cells was studied quantitatively with the dye-dilution method after intraperitoneal inoculation into mice. All irradiations were carried out in a nitrogen atmosphere. Growth curves obtained after different X-ray doses were compared to those of nonirradiated tumor cells. With 1,000 r or higher, the number of cells in the irradiated population was lower than the number of nonirradiated cells at all time intervals studied except during the last stage of the growth period.

To study the influence of irradiated cells on the growth of untreated ones, cells exposed to lethal doses of X rays were mixed with nonirradiated cells in two different proportions. The resulting mixtures were inoculated into mice and the growth of the cells was followed. Tumor cells damaged by 5,000 r exerted either an inhibiting or an enhancing effect upon the growth of admixed viable cells depending on their quantitative proportion. The irradiated cells inhibited the growth of a small population of nonirradiated cells inoculated simultaneously and also prevented the development of subsequently injected ascites tumor. These findings were interpreted as being due to an immunization of the host by dead or dying cells. The irradiated cells appeared to exert an enhancing influence on the growth of a large population of unirradiated cells. This observation suggested either that dying cells might release substances capable of stimulating tumor growth or that surviving cells which recovered from the effects of irradiation might later have contributed to the total number of growing tumor cells. The possible role of these effects in determining the growth characteristics of ascites tumor cells following irradiation of the whole population is discussed.

Quantitative studies on the ascites volume and the number of nontumorous cells in the peritoneal cavity showed that the inoculation of irradiated Ehrlich ascites cells called forth a prompt inflammatory response in the host.

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A Cytologic Study of the Intestinal Epithelium of the Mouse After Total-Body X Irradiation [1, 2]

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Since the early reports of Warren and Whipple (*1–3*) on the sensitivity of the intestinal mucosa to X irradiation, much attention has been focused upon this subject. Yet, only some papers give details concerning the progress of events which culminate in maximal damage and subsequent repair (*4, 5*). In this paper we report a study of: *a*) the time sequence of the development of injury and the repairs in the mucosa of the intestine from one-half hour after exposure to the time the animals die, in several series of mice of the same strain which had received lethal doses of X rays; *b*) the behavior of nucleic acids, mucus or other polysaccharides, periodic acid-Schiff-reactive substances and lipids during the development of the injury; *c*) nuclear damage in the crypts of Lieberkühn, the aberrant mitotic activity during the early periods of repair, and the repair in the crypts of Lieberkühn.

The two series of animals, irradiated at 1,000 r and 550 r, respectively, showed differences in the severity of damage to the intestine. Mice treated with 1,000 r died within 4 to 7 days, at a time when the crypts were largely repaired but when the intestinal villi were badly damaged. After 550 r, the mucosa of the intestine developed minor lesions on the second and third days. These lesions were completely repaired by the fourth day but the animals died on the tenth to twenty-first days.

Materials and Methods

The first series of experiments comprised 3 groups of 12 C3H mice each. In 2 groups the mice were 90 to 120 days of age and in the third group they were 40 to 60 days of age. All 36 mice were irradiated with 1,000 r. The radiation was delivered, using a setting of 200 kv., 20 Ma., with added filtration of ½ mm. Cu and 1 mm. Al. The dose rate at 20 cm. distance was 240 r per minute. In each group, mice were killed at ½, 2, 4, 8, 12, 20, 24, 32, 48, 72, and 96 hours after irradiation. A fourth group, of 24 mice 60 to 90 days old, was irradiated with 550 r under the same conditions. The mice were killed at ½, 1, 2, 4, 8, 12, 20, 24, 32, 48, 72, and 96 hours, and at

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[2] This work was conducted under Contract No. AT(30-1)-1123 between the U. S. Atomic Energy Commission and Brown University, Providence, R. I.

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5, 7, 9, 10, 11, 13, and 15 days after irradiation; of the remaining 5 animals, 2 recovered, 2 died on the 12th day, and 1 on the 13th day. Pieces of the intestine just distal to the pyloric sphincter, and just proximal to the ileocecal valve, and pieces of the cecum, were flushed with Ringer's solution and fixed in chilled Helly's fluid, Baker's formal-calcium and Bouin's fixative. Sections of Bouin's fixed tissues were stained with Delafield's hematoxylin and eosin, and with Heidenhain's iron hematoxylin.

For the demonstration of basophilic substances, 5 μ sections of tissues fixed in Helly's fluid were stained in 1/2,000 aqueous solutions of toluidine blue buffered to *p*H 4.0 and 5.0 with McIlvaine's buffer. Sample sections were stained in solutions buffered to *p*H 2.3, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0. Cytoplasmic basophilia was demonstrated at *p*H 4.0 and 5.0; below these values it was too weak, above them it was too intense. Control sections incubated 3 hours at 37° C. in 0.1-percent ribonuclease, the *p*H of which was adjusted to 6.0 with 1 *N* NaOH and then stained with toluidine blue buffered to *p*H 4.0 or 5.0, show that the cytoplasmic basophilia is abolished. This basophilic substance may be ribonucleic acid (RNA). For the demonstration of deoxyribonucleic acid (DNA), Helly-fixed tissues were stained with the Feulgen method.

Since sulfated acid polysaccharides stain metachromatically with toluidine blue (*6*, *7*), the metachromatic staining of mucins was studied in preparations stained at different *p*H values. Preparations stained with the periodic acid-Schiff-reagent method of McManus (*8*), with or without previous digestion with saliva or diastase, show mucous glands with exceptional clarity.

For the demonstration of lipids, tissues were fixed 12 hours in formal-calcium, transferred to dichromate calcium for 12 hours at room temperature (*9*) and 12 hours at 60° C., washed, and embedded in gelatin. Five micron frozen sections were colored with Sudan black.

All of these techniques were also employed in a study of the intestine and cecum in a large number of control normal mice. These observations served as our reference points for the study of X-ray damage.

Observations

The Intestine of Normal Mice

In order to make a better appraisal of the changes that develop after exposure to X rays, it seems profitable to summarize first our observations on the duodenum, ileum, and cecum of normal mice.

The small intestine of the mouse is not clearly divisible into duodenum, jejunum, and ileum. The duodenum proper, the submucosa of which contains clusters of Brunner's glands, extends for only 5 to 10 mm. beyond the pyloric sphincter; for a distance of about 30 mm., however, the proximal segment of the small intestine has long, narrow villi, which are 3 to 4 times the length of the crypts of Lieberkühn, and must be considered the duodenum (fig. 5). In the terminal segment of the small intestine, the ileum, both the crypts and the villi are shorter than those of the duodenum, although the ratio of the length of the villi to the crypts is essentially the

same as in the duodenum. In the cecum the crypts are shallower still, and the short, stout villi are usually about the same length as the crypts.

The wall of the small intestine of strain C3H mice contains large lymphoid nodules that are visible to the naked eye. The nodules also displace the mucosa luminally and the epithelium of the crypts over them usually shows a moderate amount of lymphocytic infiltration. The small intestine of strain C57BL mice shows no lymphoid nodules.

The fundus of each crypt of Lieberkühn in the entire small intestine is lined by Paneth cells (figs. 6 and 7). These cells are laden with coarse, spherical granules which stain black with Heidenhain's hematoxylin. There are no cells of Paneth in the cecum.

The epithelial cells of the villi in the duodenum are tall-columnar (fig. 4); they have a distinct cuticular border (fig. 10) and terminal bars. Toward the distal segment of the ileum the cells become gradually low-columnar. Goblet cells are numerous both in the walls of the crypts and in the villi of the entire small intestine (figs. 6, 8, 9, and 11). The epithelial cells at the apex of the villi may normally have vacuolated cytoplasm and pyknotic nuclei. This is particularly noticeable in the duodenum.

The entrance of the ductus choledochus in the duodenum is festooned by epithelial folds. Couched among the cells of the simple columnar epithelium are many goblet cells. The cytoplasm in most of the epithelial cells is riddled with vacuoles above the nucleus.

Staining with toluidine blue.—After staining with toluidine blue, the mucoid droplets in the cells of Brunner's glands in the duodenum and the granules in the cells of Paneth in the crypts of Lieberkühn remain unstained (figs. 1 and 2). The cells in the wall of the crypts have strongly basophilic cytoplasm (figs. 1 and 2); their large ovoid nuclei have a dense nuclear membrane, coarse chromatin granules and one or more nucleoli.

In the cells of the villi, the cuticular border remains unstained with toluidine blue buffered to *p*H 4.0 and is barely tinged at *p*H 5.0 (figs. 3 and 4). The cytoplasm, with the exception of a narrow line beneath the cuticular border and a semilunar area above the nucleus, contains very small granules, or closely packed, delicate basophilic fibrils. The cytoplasm above the nucleus stains more deeply than that in the base of the cell (figs. 3 and 4). The spherical or ovoid nuclei are smaller and more granular than those in the cells of the crypts. The cytoplasm of the cells of the villi of the cecum is stained less intensely than that of the cells of the small intestine. The cytoplasmic basophilia described is removed by ribonuclease and probably represents ribonucleic acid.

Goblet cells in the crypts and in the villi of the duodenum stain a light blue. In the ileum they stain more darkly, and rarely have some tinge of metachromatic staining. In the cecum, goblet cells also stain blue, but some of the larger ones have a pebbling of metachromatic staining. The free mucus in the lumen of the crypts is usually metachromatic.

Periodic acid-Schiff test.—In the duodenum, the granules in the cells of the glands of Brunner are Schiff-reactive. The granules in the cells of

Paneth are also reactive (fig. 6), although Pearse (*10*) found them unreactive in rabbits and Lillie (*11*) weakly reactive in mice. The cuticular border in both the small intestine and the cecum is Schiff-reactive (figs. 9 and 10) (*cf. 12*).

All the cells of the crypts of Lieberkühn in the small intestine and in the cecum contain distinct supranuclear and paranuclear Schiff-reactive granules (fig. 7). In the villi of the duodenum the epithelial cells have small Schiff-reactive granules just above the nucleus and underneath the cuticular border (fig. 10). The cells of the ileum have larger granules and the granules in the cells of the cecum are very coarse (fig. 9). Similar granules, described in the duodenum of the guinea pig and rabbit (*13*) and in the intestine of the rat (*12*), have been interpreted as components of the Golgi element.

The number of goblet cells in the crypts and villi is variable. Goblet cells are more numerous in the ileum than they are in the duodenum and more numerous still in the cecum. From a total of 10,000 cells in each case, counting all of the cells in well-oriented crypts and villi, goblet cells in the duodenum average from about 11 percent of the total cell population in the crypts to 8 percent in the villi.

Sudan black.—The cells in the crypts of Lieberkühn contain delicate supranuclear lipid granules. Lipid inclusions are more abundant and distinct in the epithelial cells of the villi (fig. 11). In the duodenum and ileum, with the exception of the cuticular border and a narrow band of cytoplasm beneath it, the cytoplasm of each cell is diffusely sudanophilic. Just above the nucleus, and often descending along its sides, each cell has a cluster of lipid granules (fig. 11), the location and configuration of which correspond to the presumptive Golgi area and they must be the "lipochondria" (*14*). Variations in the over-all abundance of sudanophilic elements in different specimens may reflect the amount of lipid ingested by the animal (*15*) but the lipochondria seldom change. At the tips of the villi, the extrusion zones (*16*), the vacuolated cells show neither cytoplasmic lipid, nor lipochondria. The epithelial cells on the villi of the cecum show barely visible lipochondria.

The tall, columnar cells of the ductus choledochus and the pancreatic duct contain very large lipid globules (fig. 12). In some cells, the distribution of lipids resembles that seen in the cells of the villi of the small intestine.

The Intestine of Mice Irradiated With 1,000 r

After total-body irradiation with 1,000 r, strain C3H mice appear physically normal until 48 hours, although they lose some weight. On the third day their fur becomes ruffled, their backs are arched, and they have diarrhea. On the fourth day the symptoms are accentuated, and the feces are bloody. The mice die between the fourth and the seventh day.

The earliest morphologic damage appears in the nuclei of the cells of the crypts of Lieberkühn (*1–4, 17–22*). The cells of the villi begin to show progressive degenerative changes only when the peak of damage has been

passed in the crypts. The following descriptions trace the chronology of events in the development of damage and repair.

Nuclear and cytoplasmic changes.—After exposure to X rays there is cessation of mitosis in the cells of the crypts of Lieberkühn. The ensuing damage, during the first 48 hours after irradiation, is associated with the cells in the crypts; those which cover the villi show no damage. The glands of Brunner show no detectable damage for the duration of these experiments, although Pierce (*23*) reported some damage in them.

Half an hour after irradiation, nearly all of the nuclei in the crypts of Lieberkühn are slightly enlarged. In preparations stained with toluidine blue, the nuclear membrane is thinner and less basophilic than in the normal intestine. Each nucleus contains much enlarged, basophilic nucleoli and scattered, fragmented chromatin. Small, spherical elements resulting from karyolysis are stained a dark blue. In sections previously digested in ribonuclease, toluidine blue stained neither the cytoplasm nor the nucleoli.

In Feulgen preparations, the nuclei, unlike those in the crypts of normal animals (figs. 14 and 15), are pale, with small, faintly reactive granules and rodlets scattered among the unstained nucleoli [in agreement with Warren *et al.* (*22*)]. Each nucleolus is accompanied by one or more large reactive granules. The dense karyolytic bodies are intensely Feulgen-reactive and show one or more unstained vacuoles.

One hour after irradiation little change has occurred beyond that described for ½ hour but after 2 hours there is an accentuation of the damage. In toluidine-blue preparations, the nuclei are still larger and vacuolated, the nuclear membrane is thinner, the enlarged nucleoli are deeply basophilic, and karyolysis is more advanced. The cytoplasm shows some reduction in basophilia. After digestion in ribonuclease, the basophilia of the cytoplasm and that of the nucleoli is abolished; the nuclear fragments in the cytoplasm and the spherical karyolytic bodies remain unaltered. Both the nuclei and the cytoplasm of the epithelial cells of the villi appear normal. In Feulgen preparations, the vesicular nuclei in the cells of the crypts are colored a pale pink. Fragments and clumps of chromatin, and the spherical karyolytic elements are strongly reactive.

After 4 hours many of the nuclei in the cells of the crypts are so large and vesicular that little of the surrounding cytoplasm is visible. When stained with toluidine blue, the nuclei appear as large uncolored bags that enclose faintly stained nucleoli and chromatin. Karyolysis and karyorrhexis are more abundant than in the earlier hours. The nuclei of Paneth cells appear normal and are stained fairly intensely. Cytoplasmic basophilia is nearly all gone. The nuclei are lightly Feulgen-reactive but the vacuolated karyolytic elements are strongly colored. The cells of the villi show no change.

At 8 hours the nuclei of the crypts show progressively increased ballooning, karyolysis, and karyorrhexis. There are no qualitative differences in tissue taken after 4 hours.

Mitosis reappears in the crypts 12 hours after irradiation but mitotic figures are few and abnormal (*cf. 4*). There is clumping, lagging and swelling of chromosomes, and uneven division. In some cells clusters of chromosomes are intermingled with Feulgen-reactive nuclear fragments. The cytoplasm of the cells of the crypts is only slightly basophilic. The cells of the villi show no degenerative changes.

After 20 and 24 hours, karyolysis and karyorrhexis continue in the cells of the crypts. Some karyolytic bodies are in the form of short, stout rods that resemble chromosomes. Nearly all of the intact nuclei are ballooned (fig. 24). Cell division increases appreciably, but all mitotic figures are abnormal (figs. 16 to 24). Division is unequal—chromosomes lag in the interzonal spindle, while others are scattered outside of the spindle in the surrounding cytoplasm. In some cells, chromosomes are clustered around karyolytic bodies. A rare cell in the crypts has a fairly compact nucleus and strongly basophilic cytoplasm. Some of the nuclei of the cells of Paneth have become enlarged but the cells appear normal. The cells of the villi appear to be normal.

Thirty-two hours after irradiation the crypts in the small intestine are shallower than those of normal animals. The cytoplasm of the cells of the crypts stains only slightly with toluidine blue (fig. 13). The epithelial cells of the villi show no damage.

At 48 hours the crypts are very shallow. Mitotic activity has increased but is still aberrant. Degenerative changes in the crypts have passed the peak and reparative processes are setting in. The nuclei of the cells that cover the distal portion of the villi, which until this time were approximately of the same size, now show a disparity in size, some being twice the diameter of others. The enlarged nuclei are weakly Feulgen-reactive (figs. 25 to 28). The cytoplasm of the cells with enlarged nuclei remains practically unstained with toluidine blue. The columnar cells at the base of the villi, however, still appear normal.

After 72 hours (fig. 29) the crypts are very shallow, although many of the cells have a densely stained nucleus and basophilic cytoplasm. There is still some karyolysis and abnormal mitosis. Some crypts are completely collapsed. The cells that cover the distal half of the villi are distorted, the cuticular border is gone, the cytoplasm is vacuolated and has lost most of its basophilic material (fig. 33). Some nuclei are enormously enlarged and achromic, some undergo karyolysis, and a few are pyknotic. Many cells seem to flow together, forming amorphous, multinucleated masses (fig. 32).

After 96 hours many crypts appear relatively normal, and have attained a moderate length. The cytoplasm of their cells is strongly basophilic, the nuclei are dense and ovoid, mitotic activity is abundant, and mitotic figures seem to be normal. Scattered among these repaired crypts are the remains of hopelessly damaged ones. The degree of repair is variable and repair is nearly complete only in some animals. Damage and destruction in the mucosa of the villi is maximal (figs. 30, 32, and 33). Most of the villi are collapsed and partially denuded. The remaining

epithelial cells are large, misshapen, riddled with vacuoles and they are often multinucleated. The cytoplasm of these cells is no longer basophilic.

The cells of the glands of Brunner give no indication of damage for the duration of these observations.

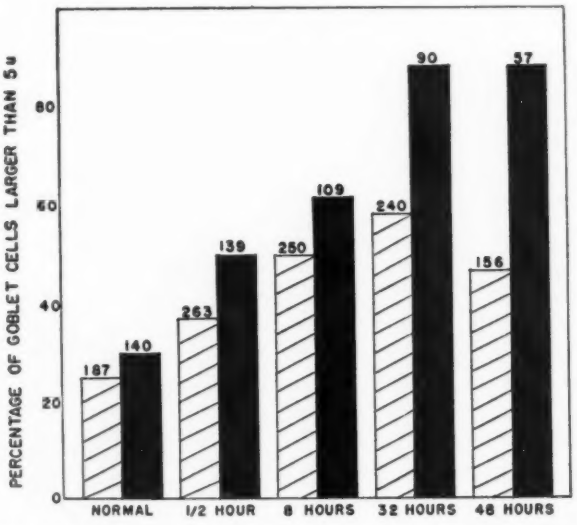
Goblet cells and "mucoid" changes.—The mucous cells in the small intestine of the normal mouse stain a light blue with toluidine blue. In the cecum, goblet cells are faintly metachromatic. All mucus is very reactive to the periodic acid-Schiff technique and even small amounts of it are detected clearly with this method (figs. 7, 9, 10). The staining properties of mucous cells in the gut show no perceptible changes from ½ to 8 hours after X irradiation. At 8 hours the mucous cells in the duodenum appear normal, but in the ileum, and particularly in the cecum, some goblet cells stain metachromatically. The mucus secreted free in the lumen of the crypts of the cecum is always metachromatic. The cytoplasm in some of the damaged cells in the crypts of the small intestine shows diffuse but distinct metachromatic staining. After the periodic acid-Schiff method the cells in the duodenum and ileum show no change, but the surface epithelial cells in the cecum show an increase in the number of supranuclear and paranuclear Schiff-reactive granules. The cytoplasm between the nucleus and cuticular border becomes Schiff-reactive (fig. 34).

From 20 to 32 hours after irradiation, there is no change in the stainability of the mucous cells of the duodenum. In the ileum, nearly all of the mucous cells in the crypts have a light metachromatic stain, but those in the villi are still orthochromatic. In the cecum, with the exception of the few goblet cells at the surface, all mucous cells stain metachromatically. Most of the degenerating cells in the fundus of the crypt of the ileum and cecum stain metachromatically and they are reactive to the periodic acid-Schiff test. Two things are apparent from these observations: *a*) the mucous cells in the duodenum are seldom stained metachromatically, even in the last stages of damage; *b*) in the ileum, and especially in the cecum, more and more metachromatic staining of the mucous cells occurs as the gut shows increased damage. This is evident particularly in the crypts; the goblet cells on the villi continue to stain mostly orthochromatically.

At 48 hours after irradiation and continuing to 96 hours, the degenerating cells of the villi accumulate granules and globules which are barely stainable with toluidine blue but which are reactive to the periodic acid-Schiff test (figs. 30 to 34).

Although it appears that there is an increase in the number of goblet cells in the intestine after 48 hours, there is actually a decrease. In the normal duodenum, goblet cells comprise about 11.0 percent of the cells of the crypts and 8.0 percent of those in the villi. There is no important change in the number of goblet cells until 48 hours after irradiation at which time they drop to 6.0 percent in the crypts; in the villi the number of goblet cells remains normal. After 96 hours the reconstituted crypts show about 4.0 percent goblet cells. At this time, the villi were so damaged that it was not possible to make careful counts.

To ascertain the changes in size which occur in goblet cells after irradiation, we have measured the greatest transverse diameter of all of the goblet cells in 30 villi and 30 crypts, in the duodenum of normal and of irradiated mice. The measurements are tabulated in text-figure 1. In the villi of the normal duodenum, the diameter of goblet cells measures 1.5 to 6 μ; 25% of these cells are larger than 5 μ in diameter and 75% smaller. In the crypts, the diameter ranges from 3 to 8 μ with 30% greater than 5 μ and 70% smaller. One-half hour after irradiation the diameter of goblet cells in the villi ranges from 2.5 to 7.5 μ with 37% greater than 5 μ and 63% smaller. In the crypts, the cells range from 3 to 7.5 μ with an equal distribution of cells greater and smaller than 5 μ. After 8 hours the cells in the villi range from 3 to 7.5 μ with an equal distribution of those greater and smaller than 5 μ. In the crypts, the cells range from 3 to 8.5 μ with 61% greater than 5 μ and 39% smaller. After 32 hours the cells in the villi range from 3 to 7.5 μ with 58% greater than 5 μ and 42% smaller. In the crypts, the cells range from 4 to 9 μ with 88% greater and 12% smaller than 5 μ. After 48 hours, goblet cells in the villi range from 3 to 7.5 μ with 47% greater than 5 μ and 53% smaller. In the crypts they range from 4.5 to 10 μ with 88% greater and 12% smaller than 5 μ. These figures show that in the normal intestine the goblet cells in the villi and in the crypts are nearly the same size, with those in the crypts averaging slightly larger. After X irradiation all the goblet cells become progressively larger, particularly those in the crypts.



TEXT-FIGURE 1.—Increase in the size of goblet cells in the duodenum after irradiation. ▨=Goblet cells in the villi; ■=goblet cells in the crypts; and the number on top of each bar indicates the total number of goblet cells measured in 30 villi or crypts.

The cells of Paneth.—Although the cells of Paneth in the human intestine are said to be sensitive to X rays (*23*), in the mouse they are fairly resistant. Until 48 hours after irradiation, the cells of Paneth show no detectable damage (figs. 6, 7). Their cytoplasm remains normally basophilic and the nuclei remain small and fairly compact. At 72 hours, however, the cells become fragmented and their granules can be seen in the lumina of the crypts. After 96 hours, the repaired crypts have a normal complement of Paneth cells, although these cells contain only a few, delicate granules.

The distribution of lipids.—During the first 48 hours after irradiation the distribution of lipids remains apparently normal (fig. 35). The cells of the crypts which are undergoing degeneration show only a diffuse sudanophilic coloration in the cytoplasm.

In the duodenum, beginning after 48 hours, the enlarged cells at the tips of the villi have a stippling of lipids throughout the cytoplasm. In the cells along the sides of the villi lipid distribution remains normal. At 72 hours, the distal ½ to ⅔ of the villi is covered by distorted epithelial cells which are engorged with lipid droplets of varying sizes (fig. 36). In the ileum, only a few of the cells have an increase in demonstrable lipid. At 96 hours nearly every cell covering the villi is grossly distorted and is laden with lipids (fig. 37). The ileum begins to show an increase in sudanophilic lipids after 72 hours and becomes more pronounced at 96 hours (fig. 38). In the cecum, patches of cells in the villi contain lipid after 72 hours; after 96 hours, most of the cells are full of sudanophilic droplets (fig. 39). At 96 hours the connective tissue of the gut shows many lipid-laden macrophages. The cells which line the crypts, even those which show obvious necrosis, seldom show appreciable increase in lipid.

Animals Irradiated With 550 r

Animals that have received a total-body irradiation of 550 r show none of the early clinical symptoms demonstrated by those irradiated with 1,000 r. They remain in apparent good health, although they lose weight daily, until the 10th or 12th day when some of them attain a rough coat and humped back. Death occurs in some animals from 10 to 21 days with peaks between 10 and 12 or between 16 and 21 days. We have observed in a large number of animals that this dose is close to the LD_{50} at 30 days for this strain and age of mice.

Nuclear and cytoplasmic changes.—During the first 2 hours after irradiation, mitosis has been arrested but the nuclei in the cells of the crypts show very little damage. After 2 hours the nuclei are slightly swollen and some karyolysis is seen. After 4 hours karyolysis and nuclear vesiculation are more advanced but mitotic activity has reappeared. These changes advance after 8 hours but the damage is not severe. Mitosis, not abundant, is present in the crypts of all 3 segments of the intestine studied. Mitotic figures show lagging chromosomes and some clumping, but none of the aberrations are so conspicuous as those seen in animals receiving 1,000 r. Some karyolytic changes continue at 12 hours.

Karyolysis and nuclear vesiculation are particularly evident at 24 hours, at which time the crypts are somewhat shallower than in nonirradiated animals. During these intervals there has been a steady diminution in the cytoplasmic basophilia of the cells of the crypts. At 32 hours nuclear damage subsides and only traces can be seen at 48 hours. At this time a few Paneth cells are fragmented. By 72 hours most of the crypts appear normal, and mitosis in them is abundant. All the animals killed after this time, including the moribund ones killed at 10, 12, and 15 days, show an apparently normal intestine.

In the cells of the villi the only nuclear damage appears at 48 and 72 hours. The nuclei in the cells in the distal part of the villi become pyknotic or swollen and hypochromic. There is no other apparent nuclear damage after this.

The number of goblet cells in the crypts remains constant until after 48 hours when it drops to about 6.0 percent; in the villi the number is constant until 72 hours when it drops to 5.0 percent. Before and after these periods, the number of goblet cells is normal.

Lipids.—The distribution of lipids in the cells of the crypts and of the villi remains normal until 48 hours. The distal part of the distorted cells at the apices of the villi of the duodenum shows a peppering of sudanophilic granules. In the rest of the epithelial cells of the villi the lipochondria become coarser than in normal cells (fig. 40). The ileum and the cecum show none of these changes. At 72 hours the epithelial cells at the apices of the villi of the duodenum show an increase in sudanophilic lipids (fig. 41). In the ileum, the few enlarged cells at the tips of the villi are free of lipid. In the cecum, scattered patches of surface epithelial cells contain coarse lipid granules. After this time lipid distribution is normal (fig. 42).

Discussion

Following different lethal doses of X irradiation there is a bimodal distribution of deaths, with peak frequencies on about the 6th and 11th days in rats (*24*) and 3½ and 11½ days in mice (*25*). Mice die from acute intestinal radiation death after 3½ days (*25*). Rats in which the whole body was irradiated but the intestine shielded also die of acute radiation death (*24*). This type of death, then, is not necessarily due to direct damage to the intestine. Certain measures outlined by Jacobson (*26*) enhance the survival of laboratory animals exposed to a single lethal dose of total-body X irradiation. Principal among these measures are: *a*) intraperitoneal implants of splenic tissue; *b*) intraperitoneal injections of whole embryo suspensions; *c*) intraperitoneal or intravenous injections of homologous bone-marrow suspensions. Jacobson (*26*) suggests that there may be a humoral factor (or factors) which, when made available to irradiated animals, brings about recovery.

In our laboratory, mice irradiated with 1,000 r die from 4 to 7 days later from acute intestinal radiation death, at a time when most of the crypts are repaired but the villi show maximal damage. Animals irradi-

ated with 550 r die after 10 to 21 days with peaks between 10 and 12, and 16 to 21 days. At these times the intestine appears normal.

Most authors agree that in the intestine of laboratory animals and in man X-ray damage occurs first in the cells of the crypts and later in the cells of the villi (*1–4, 11, 17–21, 23, 27–31*). Our observations suggest that although the cells of the villi perish, their life span is not affected by X rays.

An explanation of the damage to the cells of the villi might be found in the mode of replacement of cells in the normal intestine. The cells that cover the villi, being mitotically inert, arise from the mitotically active cells in the crypts of Lieberkühn (*32*). In the intestine of the rat, the time required for a renewal of the epithelium of the villi is on an average 1.35 days. This, then, is the life span of each cell from the time it begins its ascent at the base of the villus to the time it is sloughed off at the "extrusion zone" at the tip of each villus (*16*). The life span of the cells of the villi of the mouse is probably similar to that of the rat. After exposure to 1,000 r, mitotic activity in the cells of the crypts stops. Although mitosis reappears 12 hours later, during the next 36 hours, it is too scant and abnormal to keep pace with the cells which are degenerating. The increase in mitotic activity 72 and 96 hours after irradiation coincides with the reparative processes in the crypts. In contrast, the cells of the villi show no visible injury until after 48 to 96 hours, when they become misshapen, show a decrease in nucleic acid, become laden with lipid and are finally sloughed off. When the epithelium of the villi degenerates the crypts have been repaired (*4, 29*). If the normal life span of the cells of the villi is about 1.35 days (*16*), their subsequent degeneration after 48 hours is not likely to be due only to the effect of X ray. When the crypts are damaged the cells of the villi are not crowded out by new cells arising from the crypts and they must die *in situ*. In the villi degeneration of cells begins first at the tips of the villi; later degeneration descends halfway down the sides of the villi and after 96 hours all the cells in the villus are degenerating. This pattern of degeneration is reminiscent of the sequence of normal replacement and the older cells perish earlier. Similar, but not identical, type of damage has been obtained in the intestine with radiomimetic poisons and after starvation (*33*). The disparity in the severity of the damage in the different segments of the gut may reflect differences in rates at which the cells of the villi are normally replaced. Damage is greatest in the duodenum, less in the ileum, and still less in the cecum.

Ionizing radiation alters the metabolism of nucleic acid (*34*), X and *gamma* irradiations are said to inhibit the synthesis of DNA and to cause an accumulation of RNA in the cytoplasm (*35, 36*). In the intestine, however, an immediate rapid decrease of RNA and DNA has been reported (*5*). If one assumes that the basophilic substance which is eliminated by ribonuclease is RNA, we find a steady decrease of ribonucleic acid in the cytoplasm of the cells of the crypts until after 48 hours. The nucleoli, which become enormously enlarged, seem to show an increase in RNA. When the crypts are repaired, their cells again abound in RNA.

The basophilic staining of the cytoplasm of the cells of the villi remains unchanged for 48 hours. When the cells begin to undergo degenerative changes, all RNA seems to be lost. During the first 48 hours the Feulgen staining of the nuclei in the cells of the crypts progressively decreases (*37*, *22*). The fading Feulgen staining, however, could be the result of a dilution of DNA brought about by the many-fold increase in the volume of the nuclei. All remaining nuclear fragments are strongly Feulgen-reactive. These observations indicate a rapid decrease in RNA and a gradual loss of DNA. The loss of DNA could be due to the total destruction of cells.

The cells of Paneth remain apparently undamaged during the first 48 hours. They degenerate after 72 hours and their fragments are extruded into the lumen of the crypt. Normal Paneth cells, however, are again found after 96 hours. In the mouse, then, Paneth cells are injured much later than those in the rabbit which lose their staining properties 2 hours after total-body irradiation with 800 r and have irregular granulation until 9 days (*4*, *23*).

It has been stated that low doses of X-ray increase the number of mucous cells in the intestine but that exposure to higher doses depletes the goblet cells of mucus (*23*, *29*, *38*). Some authors believe that X rays stop mucigenous processes altogether (*39*, *40*). Friedman (*28*), however, observes that 1,000 r exerts no adverse effect on the production of mucus in the rat, and that mucous cells ripen *in situ* in the crypts as a consequence of a transitory arrest in the migration of cells. The maturation of mucous cells in the crypts would result in the so-called mucous change, hitherto considered a form of degeneration.

In the mouse, the number of goblet cells in the crypts and on the villi remains constant as long as the epithelium remains intact. When the cells in the crypts degenerate, the goblet cells interspersed among them are also sloughed off. This brings about a decrease in the number of goblet cells, even though the individual goblet cells remain relatively unaffected. After 96 hours, only 4% of the cells of the reconstituted crypts have differentiated into goblet cells. After X irradiation, mucigen is held in the goblet cells and very little of it is secreted into the lumen of the gut. In the normal duodenum the range in the size of goblet cells in the villi and crypts is nearly equal. After irradiation there is, in the villi, a progressive increase in the percentage of goblet cells with a diameter larger than 5 μ, beginning at ½ hour and continuing to 32 hours at which time 58% of the cells are larger than 5 μ. The diameter of the goblet cells in the crypts increases suddenly after ½ hour, when 51% of these are larger than 5 μ. By 32 and 48 hours the number of goblet cells in the crypts larger than 5 μ has risen to 88%. Some of these larger cells, furthermore, may measure as much as 12 μ in diameter and they are misshapen. Thus, although the changes are less striking in the villi, the goblet cells increase in size in the villi as well as in the crypts. Concerning the mechanism of enlargement of the goblet cells, we propose that mucigen is stagnated in the cells and that it imbibes water and swells. The goblet cells in the

crypts become disproportionately large, at a time when many of the non-mucigenous cells have degenerated and no longer restrict the space in which goblet cells are normally held.

Although these observations do not help to explain the mode of bacterial invasion, the stagnation of mucigen in the goblet cells may explain why bacteria cling to the epithelium of the villi from the early hours after irradiation (*17, 39, 40*). Bacterial invasion is not an important factor in "acute intestinal radiation death" (*25*). It does, however, play a role in the slower form of radiation death which occurs after exposure to 550 r (*41, 42*). It is attractive to think that bacteria may invade the tissues by way of the transient lesions which occur at the tips of the villi from 48 to 72 hours after exposure to 550 r (*17, 43*). However, the susceptibility of mice to bacteria inoculated by mouth is greatest on the eleventh day, somewhat less on the fifth day after X irradiation, and the least immediately after irradiation (*44*). Thus, it is possible that even though the intestinal epithelium appears fully repaired and normal it offers a less effective barrier than in the intestine of nonirradiated animals.

Summary

1) Total-body X irradiation with 1,000 r stops mitotic activity in the cells of the crypts of Lieberkühn. After one-half hour most of the nuclei are enlarged and some have undergone karyolysis. By 12 hours, nuclear swelling, karyolysis and karyorrhexis are more advanced, cytoplasmic basophilia is conspicuously reduced, and many of the cells perish. Sparse and abnormal mitotic activity reappears at this time. After 48 hours the degenerative processes in the epithelium of the crypts have passed their peak, except for the Paneth cells, the nuclei of which are just beginning to be swollen; in the cells at the tips of the villi the nuclei begin to be swollen and the cytoplasm loses its basophilic staining. By 72 hours Paneth cells have fragmented and have been extruded into the lumen, and the partially repaired crypts contain a number of normal cells. The cells in the epithelium in the distal half of the villi are distorted, the cytoplasm is laden with lipid globules and periodic acid-Schiff-reactive globules; the nuclei are pyknotic or show karyolysis. Cells flow together and form multinucleated giant masses. By 96 hours many of the crypts appear to be normal, but the epithelium of the villi shows a maximal amount of damage.

The cells of the crypts, then, are extremely sensitive to X rays, whereas those on the villi are relatively resistant. The cells of the villi become damaged 48 or more hours after exposure to X rays, at a time when the cells of the crypts are being repaired. The animals die when the villi show their greatest damage, but the crypts are largely repaired. The destructive changes in the epithelium of the mucosa could, at least in part, be responsible for the death of the animals.

The goblet cells in the crypts and in the villi become progressively larger until about 48 hours after X irradiation. In the crypts of the ileum and in the cecum the metachromatic staining of the goblet cells becomes

more intense until they degenerate. X irradiation seems to hold mucigen in the goblet cells, with very little of it being secreted into the lumen of the gut. The stagnated mucigen could imbibe water and distend the cell.

2) In mice X irradiated with 550 r, the progress of the injury in the epithelium of the intestine is qualitatively similar to that in mice irradiated with 1,000 r, but the damage is less severe. After 96 hours, all the damage has been repaired and the intestinal mucosa appears normal. These animals die with bacteremia at a time when the morphology of the intestine seems to be completely normal. The transient lesions which occur in the villi from 48 to 72 hours could serve as the entryway for bacteria. The insufficiency of mucus could facilitate the invasion by bacteria.

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PLATE 139

All figures in this plate are of normal duodenum stained with 0.05 percent toluidine blue buffered to p*H 4.0.*

FIGURE 1.—Crypt of Lieberkühn to show the general topography, and particularly, cytoplasmic basophilia. × 675

FIGURE 2.—Enlarged crypt of Lieberkühn. Observe cytoplasmic basophilia. The cells of Paneth in the fundus of the crypt show intense basophilia at the base but the granules remain unstained. × 1,350

FIGURE 3.—Epithelium of villi to show general topography. × 675

FIGURE 4.—Columnar cells from the side of a villus. Observe particularly the precise distribution of cytoplasmic basophilia. The cuticular border and a band above the nucleus show no basophilia. Basal part of the cells is weakly basophilic. × 1,350

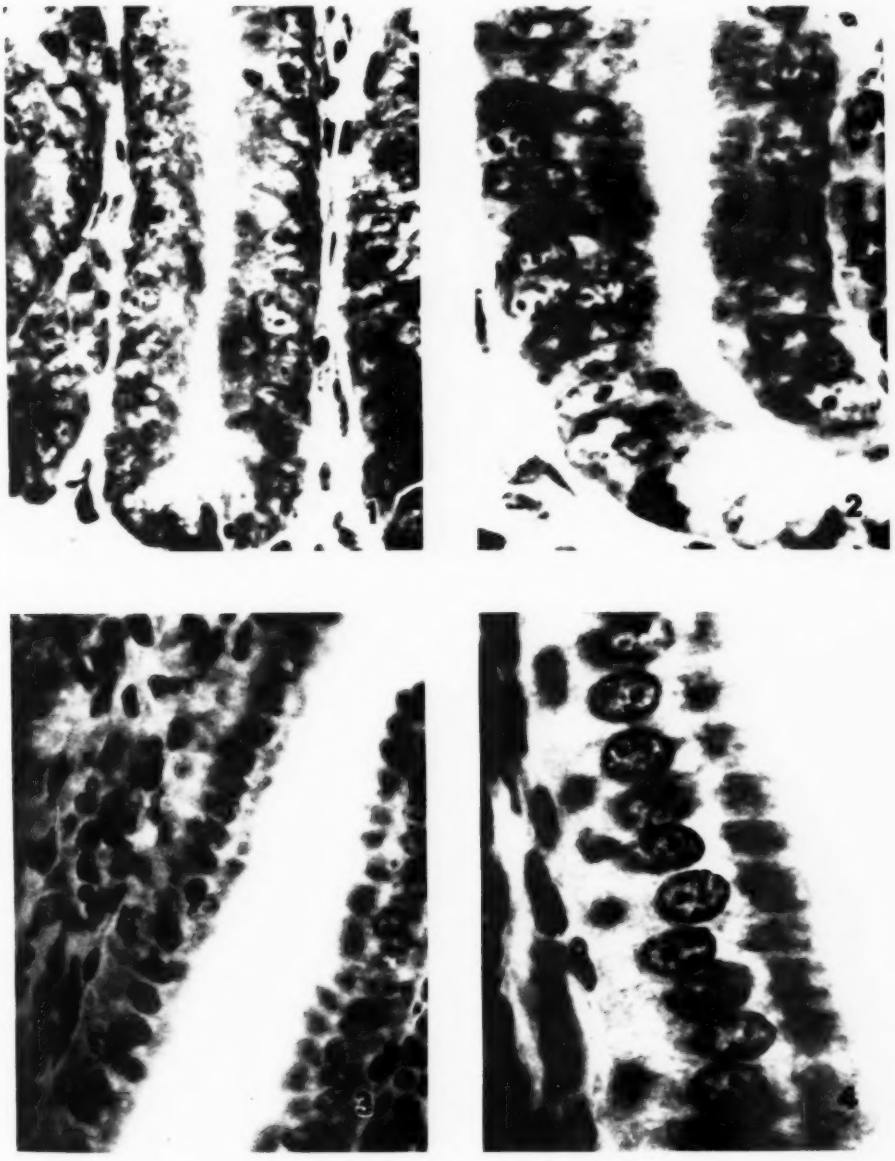


PLATE 140

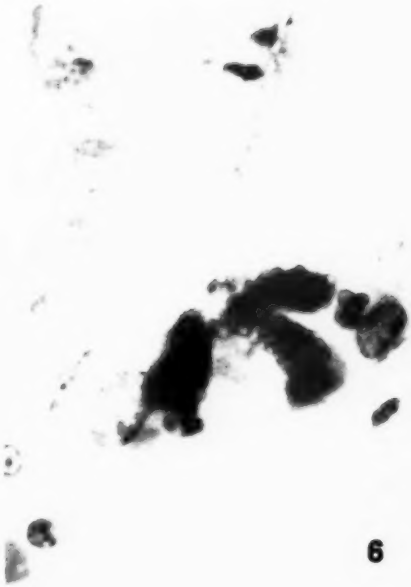
All figures in this plate are of normal intestine stained with the Schiff-periodic-acid method of McManus.

FIGURE 5.—Topographic view of duodenum. Observe particularly the distribution of Schiff-reactive goblet cells. × 125

FIGURE 6.—Cells of Paneth showing Schiff-reactive granules in the crypt of duodenum. × 1,350

FIGURE 7.—Duodenum showing the reactive granules in the supranuclear cytoplasm of crypt cells. There are two goblet cells in the upper part of the crypt and 3 cells of Paneth in the fundus. × 1,350

FIGURE 8.—Numerous goblet cells in different stages of differentiation in the crypt of the cecum. × 1,350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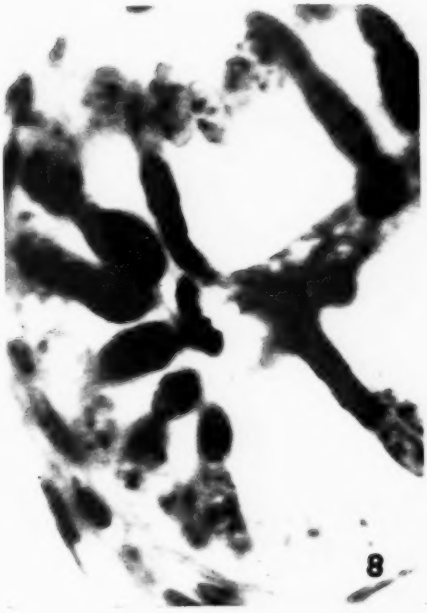
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PLATE 141

All figures in this plate are of normal intestine. Figures 9 and 10 are stained with the Schiff-periodic-acid method of McManus. Figures 11 and 12 are frozen sections colored with Sudan black.

FIGURE 9.—Normal cecum treated with Schiff reagent to show the reactive cuticular border and coarse reactive granules in the supranuclear cytoplasm of surface villous cells. × 1,350

FIGURE 10.—Cells of villus in duodenum treated with the Schiff reagent. Observe the reactive cuticular border, goblet cell and delicate reactive granules in the distal cytoplasm. × 1,350

FIGURE 11.—Frozen section of duodenum showing the characteristic distribution of lipid particles. × 675

FIGURE 12.—Frozen section of common bile duct showing abundant lipid in the epithelial cells. × 675

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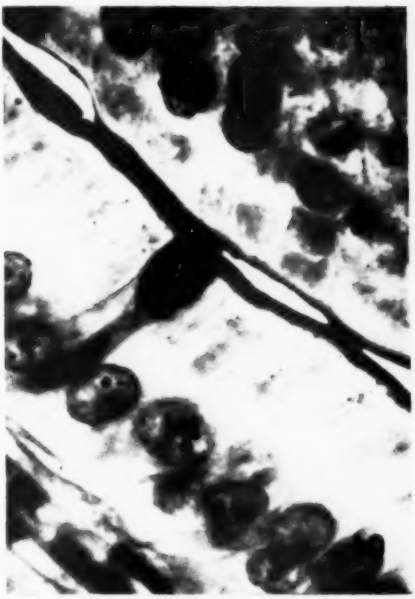

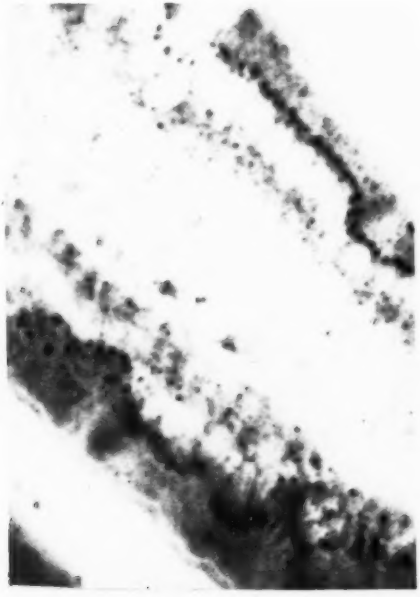

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PLATE 142

Figure 13 is stained with 0.05 percent toluidine blue buffered to pH *4.0. Figures 14 to 18 are stained with the Feulgen method.*

FIGURE 13.—Crypt of Lieberkühn in the duodenum of a mouse treated with 1,000 r total-body irradiation, 32 hours after irradiation. Compare with figures 1 and 2 and observe the fading of cytoplasmic basophilia. × 1,350

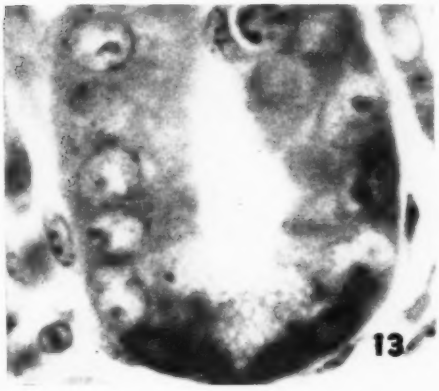
FIGURE 14.—Normal duodenum. Observe nucleus at anaphase with lagging chromosome. × 1,350

FIGURE 15.—Normal duodenum demonstrating the morphology of the nuclei in the crypt. × 1,350

FIGURE 16.—Uneven segregation of chromosomes in the crypt of the duodenum of a mouse 24 hours after irradiation of 1,000 r. × 1,350

FIGURE 17.—Observe uneven segregation in an anaphase mitotic figure, as well as several Feulgen-reactive karyolytic bodies. Duodenum 24 hours after irradiation of 1,000 r. × 1,350

FIGURE 18.—Anaphase showing the incorporation of a karyolytic body in the packet of chromosomes on the *left*. Observe also lagging chromosomes. Duodenum 24 hours after X radiation of 1,000 r. × 1,350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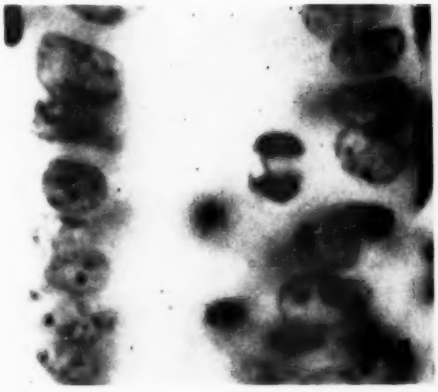

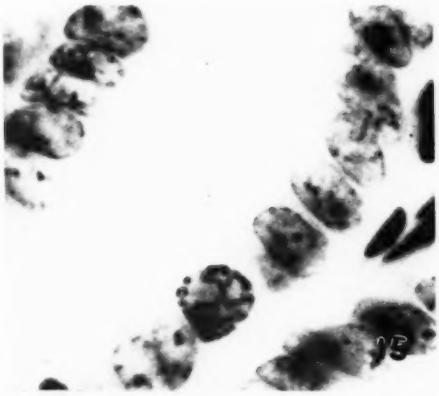

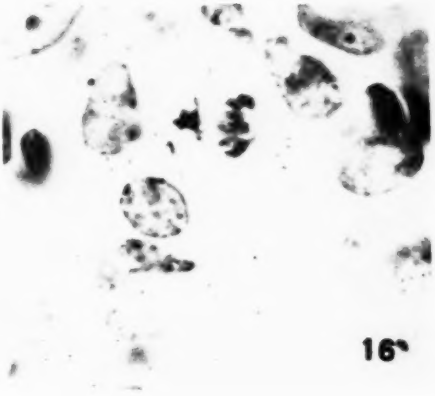
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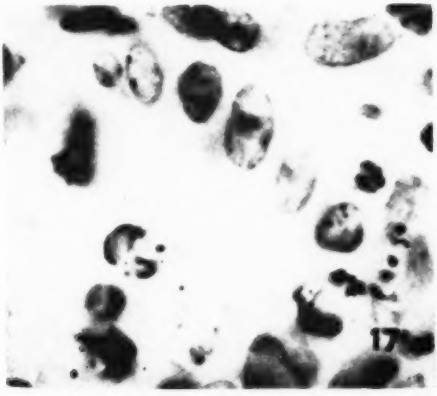
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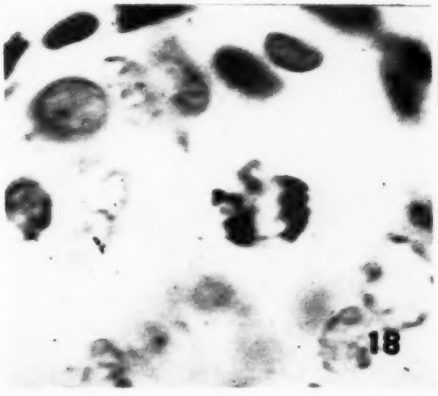
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PLATE 143

All figures in this plate are of duodenum 24 hours after X radiation of 1,000 r stained with the Feulgen method.

FIGURE 19.—Cell in metaphase showing at the upper pole some scattered chromosomes and one small karyolytic body. × 1,350

FIGURE 20.—Cell in metaphase showing the incorporation of a large karyolytic body. Observe the disparity in the diameter of the nuclei present in the field. × 1,350

FIGURE 21.—Cell in anaphase showing lagging and scattering of chromosomes. × 1,350

FIGURE 22.—Fragmented chromosomes interspersed among karyolytic bodies. × 1,350

FIGURE 23.—Chromosomes apparently being organized from karyolytic bodies. × 1,350

FIGURE 24.—Ballooned nucleus in the crypt of Lieberkühn. Note that all these nuclei are weakly Feulgen-reactive. × 1,350

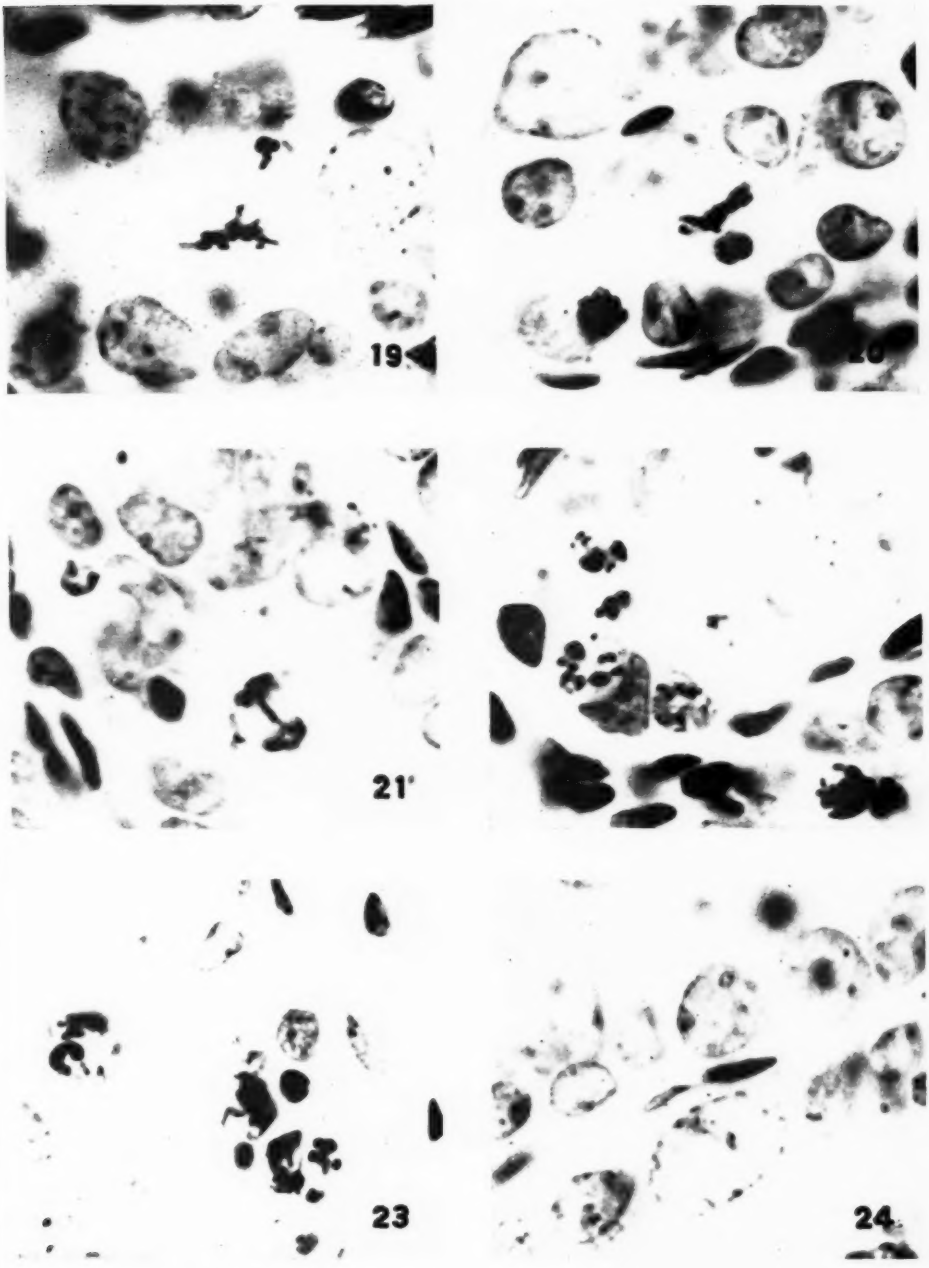
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PLATE 144

Figures 25 to 28 are of duodenum stained with the Feulgen method. Figures 29 and 30 are of duodenum stained with the periodic acid-Schiff method.

FIGURE 25.—Nuclei in surface epithelium of normal duodenal villus. Observe that the nuclei are approximately the same diameter. × 675

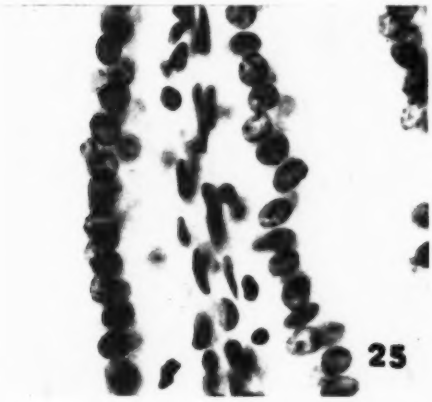
FIGURE 26.—Disparity in the size of the nuclei of surface cells in the duodenum 48 hours after 1,000 r total body irradiation. Compare with figure 25. × 675

FIGURE 27.—Higher magnification of nuclei in cells of the villus of the normal duodenum. × 1,350

FIGURE 28.—Nuclei in the duodenal villus cells 48 hours after 1,000 r total body irradiation. Compare with figure 27. × 1,350

FIGURE 29.—Topographic view of a section of duodenum 72 hours after 1,000 r total-body irradiation. Compare with figure 5. × 125

FIGURE 30.—Detail of the tip of a villus of the duodenum 96 hours after 1,000 r total-body irradiation. Observe the abnormality of the cells and the increased amount of Schiff-reactive material. × 675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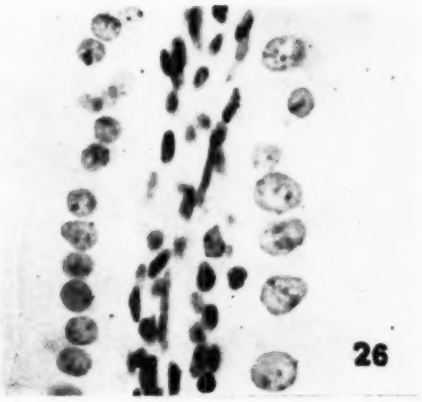
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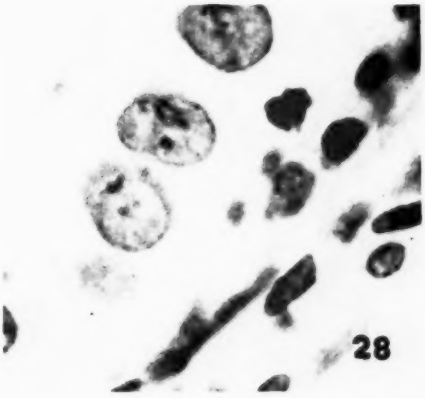
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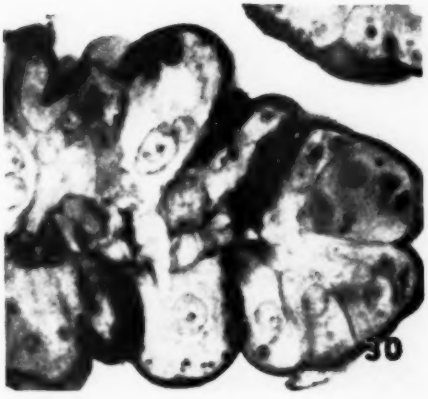
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PLATE 145

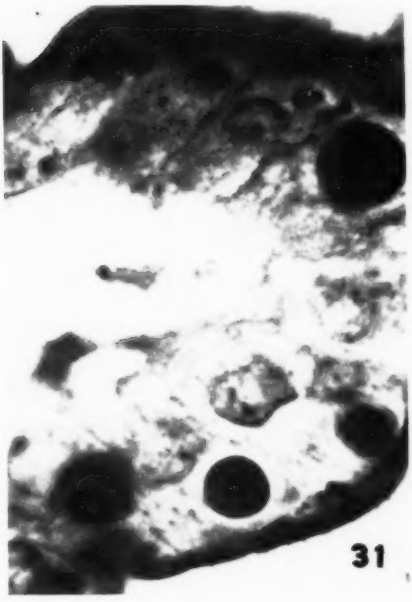
All figures in this plate are of duodenum after X radiation of 1,000 r and stained with the periodic acid-Schiff method of McManus.

FIGURE 31.—Abundant damage in the cells of a villus in the duodenum 72 hours after 1,000 r total-body irradiation. Observe the large amount of cellular aberration and pronounced increase of Schiff-reactive globules. × 1,350

FIGURE 32.—Multinucleated cell and abundant Schiff-reactive material in a villus of the duodenum 96 hours after 1,000 r total-body irradiation. × 1,350

FIGURE 33.—Degenerating, vacuolated cell in a villus of the duodenum after 96 hours. Observe abundant Schiff-reactive material. × 1,350

FIGURE 34.—Moderately damaged cells in a villus of the cecum after 96 hours. Observe the tremendous increase in Schiff-reactive material. Compare with figure 9. × 1,350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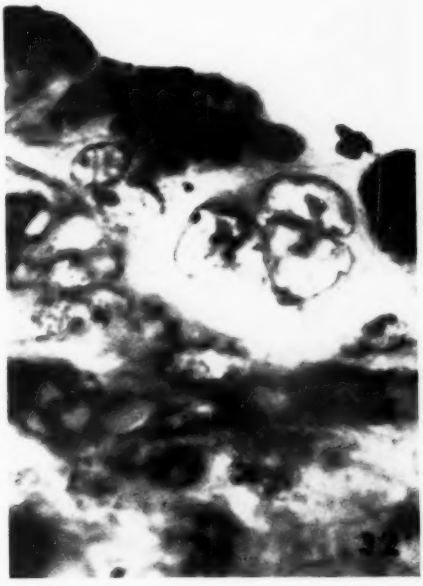
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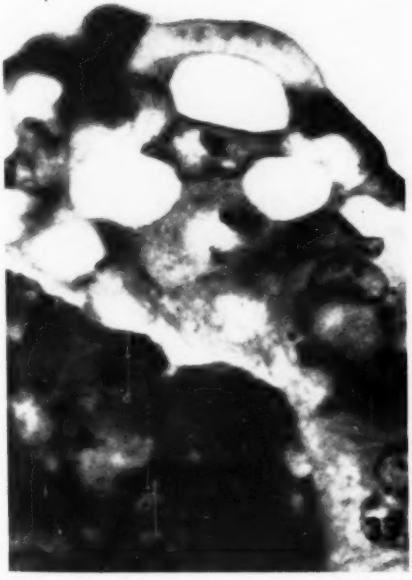
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PLATE 146

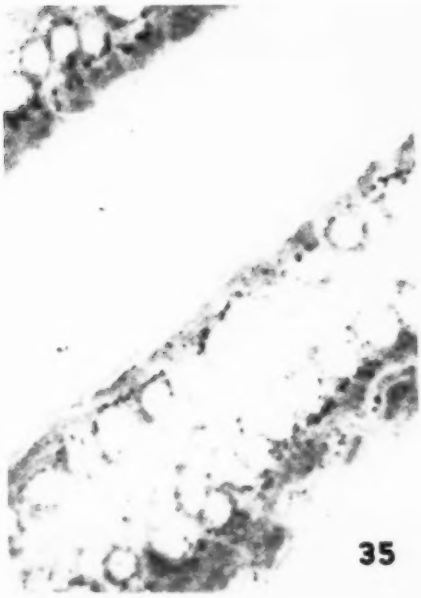
All figures in this plate are frozen sections colored with Sudan black of intestine after X radiation of 1,000 r.

FIGURE 35.—Observe the mild increase of sudanophilia in the cells of the villus in the duodenum after 48 hours. Compare with figure 11. × 675

FIGURE 36.—Lipid-laden cells in a villus from the duodenum after 72 hours. × 675

FIGURE 37.—Total lipid degeneration of the cells of the villi in the duodenum after 96 hours. × 675

FIGURE 38.—Total lipid degeneration of the cells of the villi in the ileum after 96 hours. × 675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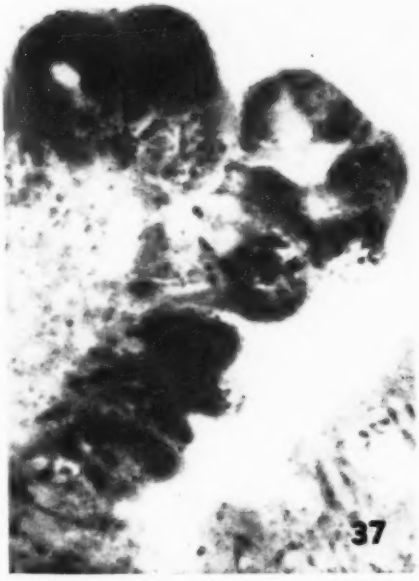
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PLATE 147

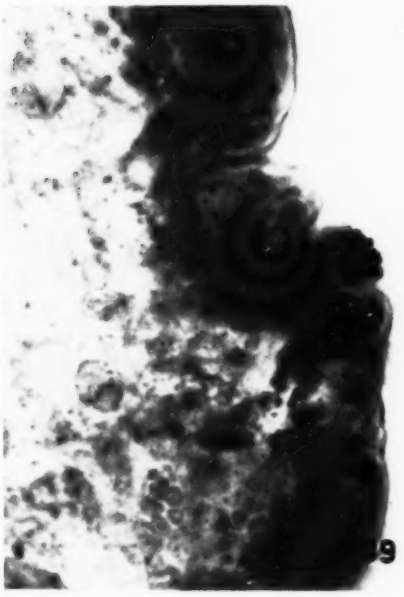
All figures in this plate are frozen sections of intestine after X radiation, colored with Sudan black.

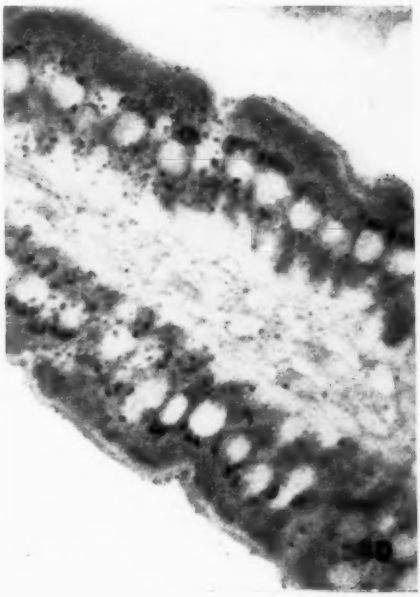
FIGURE 39.—Total lipid degeneration in the cells of a villus in the cecum after 96 hours. × 675

FIGURE 40.—Mild increase in sudanophilic granules in the cells of the duodenum 48 hours after 550 r total-body irradiation. × 675

FIGURE 41.—Maximal lipid accumulation in cells in the duodenum obtained at 72 hours after 550 r total-body irradiation. Compare with figure 36. × 675

FIGURE 42.—Lipid granules in the cells of the duodenum 96 hours after 550 r total-body irradiation. These appear normal. Contrast with figure 37. × 675





The Growth of Cells *in Vivo* in Diffusion Chambers. II. The Role of Cells in the Destruction of Homografts in Mice [1, 2]

JAMES M. WEAVER, GLENN H. ALGIRE, *and* RICHMOND T. PREHN,[3] *Laboratory of Biology, National Cancer Institute,*[4] *Bethesda, Md.*

Whether homografts[5] are destroyed by antibodies,[6] by contact with host cells, or by both has been uncertain for many years (*2*, *3*). It has been shown that vascularization is not necessary for the destruction of subcutaneous homografts, provided the hosts have been immunized to the graft (*4*). In two previous papers (*5*, *6*), evidence has been presented that mouse tissues usually survive, apparently indefinitely, in diffusion chambers placed in mice immunized against the strain of mouse from which the tissues originated. The tissues in the chambers were kept separated from the tissues of the host by filters. There was evidence that host cells did not enter these chambers. These observations raised the question whether the tissues survived because the filters provided a barrier to antibodies or because they prevented contact between implant and host cells. This question has been investigated and the results are reported in this paper.

Materials, Methods, and Preliminary Results

The preparation and assembly of diffusion chambers have been described previously (*5*). In the present experiments, "target" tissues, aseptically placed in diffusion chambers, were in all cases from mice and included: lung rudiments and embryonic hearts from 11-day embryos (C × C3H);[7] epidermal cells (actually a suspension of epidermal cells and hair follicle rudiments) from newborn C × C3H or C3H mice; and a mammary adenocarcinoma (C3HBA) from C3H adults. This tumor has been

[1] Received for publication November 8, 1954.

[2] A preliminary abstract has been published (*1*).

[3] The authors wish to thank Mr. Roy Moore, Miss Mary Borders, and Mrs. Belle Anderson for technical assistance.

[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[5] Homografts are grafts between animals of the same species, but of different genotypes. An autograft is a graft involving one animal. Isografts are grafts between two animals of the same genotype. Heterografts are grafts between animals of different species. Terms homologous, autologous, isologous, and heterologous are used to denote similar relations between a tissue and an animal or between two tissues.

[6] "Antibodies" in this paper means circulating antibodies not carried in or attached to cells, unless otherwise indicated.

[7] The following abbreviations have been used: C3H for C3H(HeN); C for BALB/cAnN; C × C3H for F_1 hybrids of these two strains; and, C57BL for C57BL/6JN.

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described by Barrett, Deringer, and Dunn (*7*). It does not grow progressively in C57BL (*8*) or C mice (*9*). The chambers were placed intraperitoneally, usually in mice, and removed after varying periods. Sometimes two chambers were placed in each host. Occasionally they were found back to back, with their circumferences matching. When this occurred, the results were omitted unless otherwise indicated. Generally, they were fixed and stained as whole mounts by a method described elsewhere (*5*) and the cells within the chambers were examined microscopically. The viability of experimental target cells was estimated and graded by comparison with control target cells in isologous systems. Where the conditions of experimental and control target cells were the same, the target cells were graded as "0." When the target cells were all dead, this was designated as "5." Intermediate conditions were denoted by "1," "2," "3," and "4," indicating progressively increasing damage. Such target cells showing intermediate effects are referred to as "injured." Grading was done subjectively but gave consistent results on repeated evaluation.

Bacteria were found in only 1 of 739 chambers examined, and the mortality rate of mice with intraperitoneal chambers was low. Only 12 out of 606 animals died (2%). There were no serious pathologic findings attributable to the chambers in any of the other hosts.

Usually the host reaction to a chamber was a mild inflammatory response. After a few weeks, a thin, strong connective-tissue sheath, containing few or no blood vessels, surrounded the chamber. However, when large numbers of cells died at one time in a chamber, host polymorphonuclear leukocytes appeared in large numbers on the outside of the chamber and disintegrated there.

Penetrability of Chambers by Host Cells

To evaluate possible interactions of target cells and host cells in diffusion chambers, it was necessary to know whether and how host cells could enter. In general, there are two routes by which cell entry might occur—through gross defects in otherwise impenetrable filters or through filters with sufficiently large pores to permit the passage of cells.

After intraperitoneal implantation, chambers with various types of filters were examined as whole mounts for the presence of host cells in the filters and in the chambers. The results are presented in table 1. It was found that where gross defects were present the first host cells to enter diffusion chambers were polymorphonuclear leukocytes. These were followed by lymphocytes and macrophages and, finally, by fibroblasts. Erythrocytes were sometimes carried in through such defects. In the absence of defects, cells were not seen in type HA "Millipore"[8] filters 150μ thick, although, in one lot of this filter, a few host leukocytes were found just inside each filter. Cells were never seen inside type AA "Millipore" filters 30μ thick. These two filters are referred to hereafter as 150μHA and 30μAA, respectively.

[8] Source: Millipore Filter Corp., Watertown 72, Mass.

TABLE 1.—*Penetrability of filters by cells**

Filter No.	Name	Thickness (μ)	Number examined as whole mounts	Number different lots of filters examined	Number with cells in filters	Number without cells in filters
1	"Millipore" HA...	150	571	6	36 (few cells just inside filters—filters all from one lot)	535
2	"Millipore" AA....	30	605	3	0.....	605
3	Schleicher and Schüell "coarse"	125	50	2	46 (moderate number cells in filters—one lot)	4 (other lot)
4	"Millipore" AA....	150	257	3	257 (many cells throughout filters)	0

*Filters 1 and 2 were examined after implantation in mice or rats; filters 3 and 4, after implantation in mice.

Although the 150μHA and 30μAA filters did not permit cells to pass through them, many processes from host cells usually were found extending into these filters. It appeared that the majority of these processes originated from macrophages. The processes were extremely tortuous, branching, and variable in diameter, ranging from about 1.5 microns down to the limit of the resolving power of the light microscope. Those in the 150μHA filters rarely appeared to extend more than half way through the filters, but those in the 30μAA filters appeared to have passed all the way through the filters.

Sometimes, a small number of host leukocytes were seen just inside Schleicher and Schüell "coarse" filters[9] and, rarely, a few were found scattered throughout the thickness of these filters and inside chambers constructed with them.

In marked contrast to the complete, or relatively complete, exclusion of host cells from chambers by the above filters, host cells were *always* seen throughout the thickness of type AA "Millipore" filters 150μ thick (150μAA hereafter)[10] and inside chambers constructed with these filters (figs. 1 and 2). The cells in these filters were drawn out in long, tortuous shapes and were thickest around their nuclei (3 to 4 microns). Macrophages and leukocytes were always seen and sometimes erythrocytes and mast cells were also observed.

Most of the host cells entering 150μAA chambers on the first day were polymorphonuclear leukocytes. Some macrophages also entered. The number of polymorphonuclear leukocytes declined rapidly and, ordinarily,

[9] Source: Carl Schleicher and Schüell Co., Keene, N. H.

[10] When the thickness of the type AA "Millipore" filter is reduced from 150μ to 30μ, it appears that the pore size is also reduced, since AA filters of the former thickness permit the passage of cells, whereas those of the latter do not.

not many were seen after a few days. These leukocytes often degenerated in chambers. Usually, after the first day, macrophages were the most numerous cells seen. Lymphocytes were seen in moderate and rather constant numbers, after the first day or two, for about 10 days. Then they decreased in number. They were much more numerous in chambers placed in mice which had been immunized by homologous tissues. Cells which were intermediate in morphology between lymphocytes and macrophages were seen rather frequently. Multinucleated giant cells frequently appeared after about a week. Mast cells were occasionally found. Plasma cells were rare and appeared only after 2 or 3 weeks. Erythrocytes were sometimes found in areas in such chambers in large numbers.

Hosts With Intraperitoneal Diffusion Chambers

In order to test the effects of diffusible substances from hosts on target cells in diffusion chambers, target cells were implanted in chambers which excluded host cells and the chambers were placed in adult male mice of various inbred strains which were isologous, homologous and non-immunized, or homologous and immunized to the target cells. Animals were immunized by one subcutaneous injection (under the dorsal skin) of a piece of spleen (about 3 mm. in diameter from adult male mice of the same strain as the target cell) 7 to 13 days before experiments were begun. To test the effects of leukocytes and macrophages from hosts, the procedure was similar except that one or both of the filters of each chamber was 150μAA, so that these cells could enter the chambers.

Combination of Spleen Cells With Target Cells in Diffusion Chambers

In further experiments, it was desired to test the effects on targets of spleen cells from mice which were isologous, homologous, or immunized to the targets. Accordingly, target cells were combined in chambers with spleen cells from such mice. The donors of the spleen cells were adult male inbred mice. The filters of the chambers were impenetrable by cells. The genetic relationship between the hosts in which these chambers were placed and the target cells in the chambers were varied.

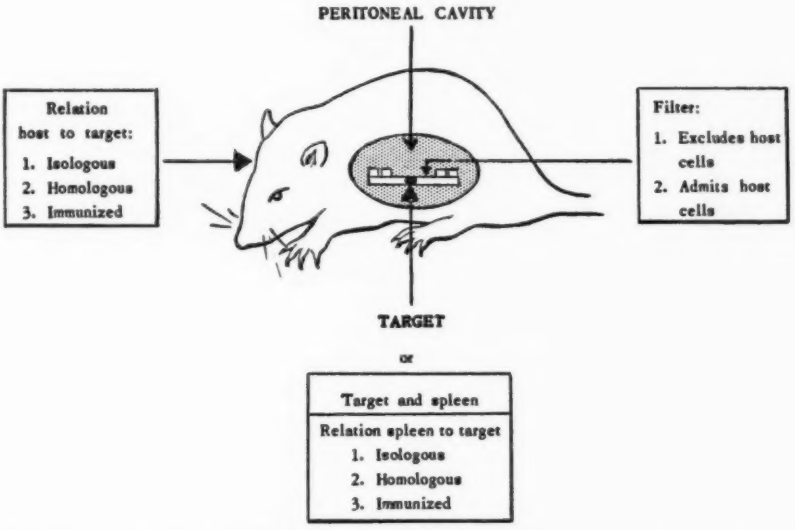
Summary of Experimental Variables

Text-figure 1 illustrates the most important experimental variables. Details of the methods of individual experiments are described with the results of these experiments.

Preparation and Implantation of Tissues in Diffusion Chambers

Lung rudiments and embryonic hearts were dissected from 11-day C $\times$ C3H embryos. Dissection was done in horse serum with dissection needles. The rudiments and hearts were handled by means of orally controlled fine pipettes. One rudiment or heart was placed in each chamber.

Epidermal cells were separated from the skins of newborn mice by means of trypsin. The method used is a modification of a method described by Medawar (*10*). The mice were decapitated and their extremities were amputated. The bodies were washed with soap and water and then rinsed several times with water. The skins were cut down the dorsal midline and peeled off the trunk. They were floated raw side down for 2 hours on a filtered 3-percent solution of *crude* trypsin in a balanced saline (3 grams of trypsin in 100 cc. of saline, then filtered through filters of decreasing porosity). A variety of different salines were used with very similar results. The skins were then placed dermis down on a



TEXT-FIGURE 1.—Diagram of experimental variables. Cross section of intraperitoneal diffusion chamber (not drawn to scale).

glass surface. A corner of the dermis of each skin was grasped with a forceps and a corner of the loose epidermis was grasped with another forceps. The epidermis was quickly pulled off and immediately placed in a centrifuge tube containing 10 cc. of horse serum and then was vigorously shaken. The epidermal cells dropped off the sheet of keratin. This sheet was discarded. This was repeated until as many epidermal cells had been collected as desired. The cells were then centrifuged at 1,100 r.p.m.[11] for 10 minutes; their volume was recorded; and they were resuspended in three times their volume of horse serum (a "25% suspension"). Two hundredths of a cubic centimeter of this suspension was used per chamber.

Pieces of the C3HBA mammary adenocarcinoma of approximately 1 mm.^{3} were placed in chambers (one piece per chamber).

[11] A Clay-Adams "Safeguard" centrifuge with a 6-tube angle-head was used for all centrifuging.

Suspensions of washed spleen cells were prepared as follows: Two or three adult spleens were placed in a 2-cc. syringe containing a stainless steel wire sieve (24 mesh/inch) at its outlet. The spleens were crushed through the sieve into a centrifuge tube containing 10 cc. of horse serum; this suspension was immediately shaken vigorously and centrifuged for 10 minutes at 1,100 r.p.m. The horse serum was decanted and the cells (about 0.2 cc.) were resuspended in 10 cc. of fresh horse serum. Centrifuging was repeated as above. Red cells comprised about half the total cell volume. The horse serum was again decanted and a few drops of horse serum added. With gentle agitation and careful pipetting, the topmost white layer of cells was pipetted off into another tube. These cells were centrifuged again as above; their volume was recorded; then they were resuspended in a volume of horse serum equal to their volume (50% suspension) or three times their volume (25% suspension).

In some experiments, a lung rudiment or a piece of the C3HBA mammary adenocarcinoma was combined in a chamber with 0.01 or 0.02 cc. of a 50-percent or a 25-percent suspension of spleen cells. In other experiments, equal volumes of a 25-percent suspension of epidermal cells and a 25-percent suspension of spleen cells were mixed and 0.02 cc. of this mixed suspension was placed in a chamber.

Morphology of Target Cells and Spleen Cells in Diffusion Chambers

Before describing the results of experiments to investigate the role of host cells in the destruction of homografts, it is necessary to describe the morphology of target cells and spleen cells in diffusion chambers, so that it may be seen how, and to what extent, these two types of cells were distinguishable from each other and from host cells entering the chambers. The types of host cells which entered chambers through defects, or through 150μAA filters, have already been described.

Target Cells

Following are descriptions of target cells in diffusion chambers placed in hosts isologous to the target cells. The chambers did not admit host cells.

Lung rudiments.—Lung rudiments were found to be the most satisfactory target cells for general purposes.

They were initially about 400 by 600 microns and consisted of a horseshoe-shaped epithelial mass surrounded by a layer of condensed mesenchyme.

After implantation in chambers, mitoses were numerous at first but became rare after 2 or 3 weeks. By then, the epithelial part of the rudiment was about 3 mm. in diameter and the mesenchyme had covered the rest of the chamber. The epithelium formed bluntly rounded lobules which often had dividing septa. Normally, or in tissue cultures (*11*), the epithelium branches into finer and finer tubes. Sometimes the lobules formed in chambers were ringed peripherally with small tubules with a few branches. Figure 9 is typical of this kind of growth. The interior

of these structures became hollow and often contained a few necrotic cells. There was little, if any, necrosis in the mesenchyme. Sometimes, the lobules were lined with alveolus-like sacs opening into a central lumen. All the epithelial cells differentiated into easily recognizable cells.

Masses of cartilage frequently developed in these rudiments. Areas of hematopoiesis and networks of empty capillaries (lymphatics?) in the mesenchyme were not uncommonly seen.

Embryonic hearts.—Whole embryonic hearts were placed in 30μAA chambers and inserted intraperitoneally. For a few days they continued beating as a mass. This could be seen when the chambers were removed from the hosts and viewed with transmitted light. But, later, such hearts became disorganized and only twiched in small areas. Cytologically, the heart cells were not particularly easily distinguishable from other types of cells.

Epidermal cells.—Epidermal cell suspensions had the advantage as target cells in chambers in that they did not contain or form leukocytes. Hence, any leukocytes seen in such chambers were derived from the host.

These suspensions of epidermal cells consisted initially of small, living hair follicle rudiments (consisting solely of epithelial cells), of small living sheets of basal cells from the epidermis, of degenerating individual basal cells, and of granular cells of the epidermis (fig. 3). Exhaustive microscopic examination always failed to show any cells of mesodermal origin in these preparations.

In chambers, mitoses were numerous for about a week and rare after 2 weeks. The cells spread out and coalesced to form a sheet. Figure 7 is typical of this. Some cells degenerated in the center of the hair-follicle rudiments. Cells frequently separated from the edge of a sheet and somewhat resembled fibroblasts. Finally, a layer of cells resembling epidermis was formed. New granular cells and keratin were produced centrally by differentiation of the cells initially in contact with the filters.

Mammary adenocarcinoma.—The C3HBA mammary adenocarcinoma grew rapidly and filled chambers in about 3 weeks. The epithelium grew in cords, sheets, and buds. Histologically, these epithelial cells were quite recognizable. The stroma was rather scanty, but a few connective-tissue cells were present. Mitoses of the tumor cells continued indefinitely and the central layers of these cells (farthest from the filters) became necrotic.

Spleen Cells

The initial suspensions of spleen cells consisted mainly of individual lymphocytes of various sizes (or closely related cells), masses of red cells (usually about 20% of the total cell volume), varying numbers of megakaryocytes, and cellular debris. A few larger fragments of spleen were sometimes present.

In chambers, mitoses began during the first day or two and became very numerous. Soon a large fraction of the population consisted of large, rounded, separate cells with large nuclei. These cells frequently were seen in division (fig. 4). It appeared that further divisions and matura-

tion of these cells led to the production of new red cells, of polymorphonuclear leukocytes, and, probably, of lymphocytes. After a few days, macrophages appeared in large numbers and frequently divided. Plasma cells sometimes appeared after about a week. After about 10 days, sinusoidal capillary networks often appeared and sometimes grew so as to fill a large part of the chamber. After a month or more, the most numerous cells were masses of fibroblast-like cells and macrophages, sometimes containing foci of granulocytes and lymphocytes. Mitoses were rare after 3 weeks.

When the number of spleen cells per chamber was increased to the maximum used (0.02 cc. of a 50% suspension), these cells rather frequently died about a week after implantation. Often, any target cells also present in these chambers died shortly afterward. The death of these cells was followed by the type of host polymorphonuclear reaction described above. Chambers in which this occurred have been omitted from the results presented in the following sections.

Results

Mouse Cells in Diffusion Chambers Placed in Immunized Mice

The purpose of these experiments was to study target cells surviving in chambers placed in mice previously immunized to the target cells (by inoculation of adult spleen from mice of the strain of origin of the

TABLE 2.—*Methods used to study mouse targets in diffusion chambers placed in immunized mice* *

Strains of hosts	Targets		Filters	Time chambers in hosts (*days*)	No. of chambers examined
	Tissues	Strains of donors			
C†	Lung rudiment	C × C3H	30μAA	9	1
				15	1
				22	1
				35	1
	Embryonic hearts	,,	,,	9	1
				15	1
C	Epidermal cells	C3H	150μHA	4	1
				34	1
				67	1
				74	1
				94	1
				214	4
C57BL	C3HBA tumor	,,	Schleicher and Schüell "coarse"	11	3
				19	2
				34	1
				47	2
				118	2
C	,,	,,	30μAA	45	5

*Immunized by C × C3H or C3H spleen before the experiments were begun.

†Experiment also done in C × C3H and normal C hosts at 9 and 15 days with 4 hearts, and at 9, 15, and 22 days with 6 lung rudiments. The results were the same as in the immunized C hosts.

target cells) in order to investigate whether or not host cells entered the chambers. The filters used were 30μAA, 150μHA, and the Schleicher and Schüell "coarse" filters. Further details of the methods are presented in table 2.

Lung rudiments and embryonic hearts from C × C3H embryos survived at least 35 and 15 days, respectively, in 30μAA chambers placed in immunized C mice. The chambers containing hearts were studied for beating and then fixed and stained. Epidermal cells from C3H mice were placed in 150μHA chambers in immunized C mice. These cells were healthy until 94 days after implantation. At this time, a few host leukocytes were observed to be inside the chamber and the number of epidermal cells was not large. Part of this chamber was tested for C3H cells by means of a test[12] devised by Merwin and Hill (*4*) (the cells in ¾ of this chamber were tested in another C host to determine whether they immunized this host against C3H cells) and the results indicated that such cells were present. Similar results were obtained at 214 days. The decrease in epidermal cells coincident with the entrance of host leukocytes suggested that the former cells were being destroyed by the latter. The C3HBA mammary adenocarcinoma from C3H mice survived in chambers with Schleicher and Schüell "coarse" filters in immunized C57BL mice for at least 118 days. Two thirds of each chamber was broken open into a C3H mouse subcutaneously and this test was considered positive if the mouse developed a tumor at this site in 3 months. The other third of each chamber was fixed and stained. A few host leukocytes were found in some of the filters and chambers. Chambers with 30μAA filters allowed implants of this tumor to survive at least 45 days in immunized C mice as judged by the morphology of the tumor cells.

There was no evidence that mitoses of target cells were inhibited in these immunized hosts.

These results are in accord with those in previous papers (*5*, *6*) in that a variety of target cells survived in chambers placed in immunized hosts, and further evidence was obtained that few, if any, host cells had entered these chambers—except as noted above.

Mouse Cells in Diffusion Chambers Placed in Immunized Rats

While it seemed unlikely, from what is known concerning the pore size of the filters used in the preceding experiments, that protein molecules the size of antibodies would have been excluded from the chambers used, it was considered important to test this point *in vitro*. It was found that equine *gamma*-globulin solutions (18 mg./cc.) passed through these filters without detectable change in concentration.[13] Since it is generally accepted that antibodies often are produced to heterografts, this problem was explored further *in vivo* as follows:

In table 3 are presented the results of experiments in which lung rudiments or epidermal cells from mice were implanted in cell-impenetrable

[12] Tests performed by Dr. Ruth Merwin, National Cancer Institute, Bethesda, Md.
[13] Analyses performed by Dr. Benton Westfall, National Cancer Institute, Bethesda, Md.

chambers which were placed in *rats* previously immunized to these target cells. Similar chambers were placed in normal rats as controls. The target cells survived in the normal rats for a week as well as they had in isologous mice in other experiments. (It is unknown how long target cells will survive in chambers placed in heterologous hosts.) However, those in immunized rats were usually injured or killed after 2 days (figs. 5, 6, 7, 8). Presumably, this was due to the passage of antibodies into the chambers. No difference was apparent in the results when thin filters (30μAA) or thick ones (150μHA) were used.

TABLE 3.—*Mouse cells C × C3H in diffusion chambers placed in normal or immunized rats**

Target tissues	Filters	Results expressed as time in days over condition of target cells (0=none killed, 5=all killed; each number represents one chamber)	
		Results in normal rats	Results in immunized rats
Lung rudiments	30μAA	3 6 7 0 0,0,0 0,0,0,0	3 6 7 2 2,2,4 2,3,3,3,4,4,5
Epidermal cells	150μHA	4 0,0,0,0,0,0	2 4 0 0,1,2,2,3,3,5,5

*Immunized by C × C3H or C3H spleen before the experiments were begun. The rats were Holtzman males, 3 to 6 weeks old.

Effects of Host Cells Entering the Diffusion Chambers Upon Target Cells Within the Chambers

To test the effect of "immunized" homologous cells on target cells in diffusion chambers, target cells of mouse tissues were placed in chambers which admitted host leukocytes and macrophages and the chambers were placed in immunized mice. Other chambers, impenetrable to cells, were also placed in such hosts. Similarly, chambers of these two types were placed in isologous or homologous mice. The methods and results of these experiments are presented in detail in table 4 and summarized in table 5.

When target cells were implanted in diffusion chambers which admitted host leukocytes and macrophages and the chambers were placed in immunized mice, most of the target cells were destroyed or injured within a week (fig. 10). As before, target cells in chambers which did not allow these host cells to enter usually survived without injury in such hosts (figs. 9, 13), although in one experiment (expt. 8), some of the lung rudiments were injured.

As was expected, target cells survived in cell-impenetrable chambers placed in isologous and homologous hosts as well as they did in immunized hosts.

Since target cells were destroyed in chambers which permitted cells from immunized hosts to enter, it was necessary to determine whether the

TABLE 4.—*Target cells in cell-impenetrable or cell-penetrable diffusion chambers*

Expt. No.	Target cells	Host mice	Results expressed as time in days over condition of target cells (0=none killed, 5=all killed; each number represents one chamber)	
			Results in diffusion chambers impenetrable by host cells*	Results in diffusion chambers penetrable by host cells†
1‡	C3H mammary adenocarcinoma §.....	C3H	9/0,0 10/0,0 11/0,0,0,0 31/0 62/0,0,0	6/0+ 9/0,0 10/0,0 11/0,0,0,0,0,0,0,0 19/0+ 31/0 62/0,0,0 90/0+
2	"	C57BL	9/0	9/0,0 15/1,2 16/0,0,0,0,1
3	"	C	9/0 12/0 16/0 23/0 25/0 31/0	9/1 12/2 16/2 23/0 25/0 31/0
4	"	C57BL previously immunized with C3H spleen.	6/0 8/0,0 9/0,0 (13/0+,0+ 20/0+,0+ 47/0+,0+)‖	3/0 6/2,3 8/3,5 9/4,5,5,5,5,5+ 13/5−,5− 19/4− 20/5−,5− 47/5−,5− 90/5−
5	"	C previously immunized with C3H spleen.	8/0 10/0,0,0 14/0,0	8/1 10/1,3,3 14/1,2 23/5,5 27/5,5 30/3,3,5,5
6	C × C3H lung rudiments.....	C × C3H	1/0 4/0,0,0 6/0,0,0 9/0,0,0 15/0 22/0	1/0 3/0,0,0 4/0 6/0,0,0
7	"	C57BL	1/0 4/0 6/0,0,0	1/0 4/0 6/0,0,0,0,0,0,0
8	" .	C57BL previously immunized with C × C3H spleen.	1/0,0 4/0 6/0,0,0,0,0,1,2	1/1,1,1 2/1 3/2,4,5,5,5,5 4/2 5/2,4,4 6/2,2,3,3,4,5,5
9	C3H epidermal cells.....	C3H	2/0 4/0,0 6/0 11/0	2/0 4/0 6/0 8/0 11/0
10	"	C	2/0 7/0 9/0 14/0 19/0	2/0 7/0 9/2 14/3 19/2 140/5
11	"	C previously immunized with C3H spleen.	1/0,0 3/0 5/0,0 6/0 7/0,0,0 14/0 19/0,0,0	1/0,1 3/0 5/2,2,3 6/2 7/3,5 14/5,5 19/5,5,5 32/5 140/5

*The filters of the cell-impenetrable diffusion chambers in experiments 1–5 and 9–11 were 150μHA. In experiments 6–8, one filter of each chamber was 150μHA and the other was 30μAA.
†The filters of the cell-penetrable diffusion chambers in experiments 1–5 and 9–11 were 150μAA. In experiments 6–8, one filter of each chamber was 150μAA and the other was 30μAA.
‡At 9, 31, and 62 days: one 150μHA and one 150μAA chamber were placed in each host and the two chambers in each host were adherent at autopsy.
§+ or −=⅔ of each chamber opened and placed in a C3H mouse; +=recipient developed a tumor; −=recipient did not develop a tumor.
‖Results from a previous paper (*5*).

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TABLE 5.—*Summary of experiments in table 4 in which chambers were in hosts 3 to 15 days*

Genetic relationship of hosts to target cells	Host cells did not enter chambers			Host cells entered chambers		
	No. of chambers		Chambers with dead target cells (*percent*)	No. of chambers		Chambers with dead target cells (*percent*)
	With no target cells killed (graded 0)	With some to all target cells killed (graded 1–5)		With no target cells killed (graded 0)	With some to all target cells killed (graded 1–5)	
Isologous....	21	0	0	24	0	0
Homologous..	10	0	0	11	4	27
Immunized homologous.	27	2	7	2	42	95

entrance of isologous or homologous cells into similar chambers had any destructive effect on target cells in these chambers.

It was clear that the entrance of isologous host cells inhibited mitoses in lung-rudiment target cells (fig. 12). However, these host cells did not *kill* any of the target cells. Similarly, isologous host cells inhibited mitoses of epidermal cells.

Homologous host cells (fig. 11), entering such chambers, also inhibited mitoses of target cells for the first few weeks and, later, occasionally killed target cells. However, this destruction was much slower and less extensive than when "immunized" cells entered.

Target Cells Combined With Spleen Cells

The data in the preceding section support the hypothesis that homografts are destroyed by the host cells which accumulate about them rather than by antibodies. However, the question may be raised whether it is possible that homografts are killed by antibodies which are unable to pass through the filters impenetrable by cells, but which can pass through filters penetrable by cells.

Therefore, the experiments to be presented in this section were performed. Washed suspensions of spleen cells from immunized mice were tested for any destructive action against homologous target cells by combining the two types of cells in cell-impenetrable diffusion chambers. These chambers were placed in mice isologous to the target cells. Antibodies to the targets cells could not have been present in these chambers unless they were inside of or tightly attached to the "immunized" spleen cells. The methods and results of such experiments using lung rudiments or epidermal cells as target cells are detailed in table 6 and summarized in table 7.

Such targets were destroyed or injured in *isologous* hosts in a week or less, when combined in chambers with a washed suspension of "immunized" spleen cells (fig. 16). Similar targets combined with homologous or isologous spleen cells were not injured in this time (figs. 14, 15).

TABLE 6.—*Combination of target cells with spleen cells in cell-impenetrable diffusion chambers**

Expt. No.	Target cells	Hosts	Donors of spleen cells (mice)†	Results expressed as time in days over condition of target cells (0=none killed, 5=all killed, each number represents one chamber)
1	C × C3H lung rudiments	C × C3H mice	C × C3H	3/0,0 5/0,0,0,0 0,0,0,0 0,0,0,0 0 9/0,0,0 0,0 10/0 15/0 21/0
2	,,	,,	C57BL	5/0,0,0,0,0,0,0,0
3	,,	,,	C	3/0,0,0 9/0,1 10/0 15/0,0 21/0,0,1,1,1,2
4	,,	,,	C57BL previously immunized with C × C3H spleen.	1/0,1 2/1,1 5/1,2,2,2,2,2,2,2,2,2,3,3,3,3,3
5	,,	,,	C previously immunized with with C × C3H spleen.	3/1,1,2,2,2,3 5/1,2,5 9/1,2,5
6	C × C3H epidermal cells	,,	C	3/0,0,0,0,0,0,0 6/0,0 16/3 34/5
7	,,	C mice previously immunized with C3H spleen	,,	3/0,0,0,0,0
8	,,	C × C3H mice	C previously immunized with C3H spleen.	3/0,2,2,3,3,3,3,3 6/0,3,5 16/5 34/5
9	,,	C mice previously immunized with C3H spleen	,,	3/1,2,2,3,5,5
10	,,	Holtzman rats	C	3/0,0,0,0
11	,,	,,	C previously immunized with C × C3H spleen.	3/3,3,4,5

*The filters in experiments 1–5 were 30μAA; in 7, 9, 10, and 11, 150μHA; and in 6 and 8, either 30μAA or 150μHA.

†The number of spleen cells per chamber in experiments 1–5 was varied as described in the section on implantation of tissues in chambers. However, the results did not vary with the number of implanted spleen cells, possibly because divisions of these cells rapidly eliminated the initial difference in numbers.

TABLE 7.—*Summary of experiments in table 6 in which chambers were in hosts 1 to 14 days*

Genetic relationship of spleen cells to target cells	Number of chambers		Chambers with some to all target cells dead (graded 1–5) (*percent*)
	With no dead target cells (graded 0)	With some to all target cells dead (graded 1–5)	
Isologous	21	0	0
Homologous	31	1	3
"Immunized" homologous	3	49	94

Essentially the same results were obtained when implants of the C3HBA mammary adenocarcinoma were combined in chambers with isologous, homologous, or "immunized" homologous spleen cells (figs. 17, 18).

Epidermal cells were combined in chambers with homologous or "immunized" spleen cells in hosts *immunized* to the target cells. The results were similar to those when the hosts were isologous to the target cells. Such results were obtained in other experiments even when the hosts were rats.

Combining target cells in chambers with homologous but "non-immunized" spleen cells resulted in some injury to the target cells. However, this injury occurred later and was not so severe as was the case when target cells were combined with "immunized" spleen cells.

Since target cells died when combined in chambers with "immunized" spleen cells, regardless of the strain of the host, regardless of whether or not the host had been immunized to the target cells, and even regardless of the species of the host, it seemed likely that target cells would die when combined *in vitro* with "immunized" spleen cells. Accordingly, epidermal cells were combined with "immunized" spleen cells *in vitro* in chicken plasma clots. The supernatant fluid was 40-percent horse serum, 40-percent Earle's saline, and 20-percent chick embryo extract. The gas phase was air and the cultures were incubated at 37° C.[14] However, no clear destruction of the epidermal cells was seen as long as the experiment lasted—5 days. Lymphocytes were seen moving about in contact with the target cells for 3 days, but these lymphocytes had largely disappeared after 5 days. Similar parallel cell combinations in chambers *in vivo* resulted, as usual, in extensive target cell death in 3 days.

Details of the Destruction of Target Cells in Diffusion Chambers

Lymphocytes and/or macrophages from immunized hosts were the cells always associated with target cells undergoing destruction in diffusion chambers. Plasma cells from such hosts appeared to be too infrequently present to be involved. On the other hand, macrophages from such hosts were frequently found in contact with target cells without evidence of

[14] Further details of the method of culturing the epidermal cells will be published subsequently. Tissue culture facilities supplied by Dr. Wilton Earle and Dr. Virginia Evans, National Cancer Institute, Bethesda, Md.

destruction of the latter cells. Whenever target cells were found together with lymphocytes from mice immunized to the target cells, both types of cells were degenerating or dead. If the two types of cells were slightly separated, both were healthy. Lymphocytes were sometimes found inside of large target cells—both target cells isologous to the lymphocytes and those to which the lymphocytes had been "immunized." In both cases, the lymphocytes degenerated. However, the target cells involved died only if the lymphocytes had been "immunized" to them. Target cells dying in association with "immunized" lymphocytes were seen in a variety of forms of degeneration. They were not killed by being phagocytized. Frequently, no phagocytic cells were nearby.

There was similar evidence that the lymphocyte was the cell responsible for the slower destruction of target cells by homologous but "nonimmunized" cells.

After target cells and "immunized" lymphocytes in contact had begun to degenerate, target cells and lymphocytes nearby, but not in contact with each other, also died. Examples were seen when pieces of the C3HBA mammary adenocarcinoma were combined in chambers with suspensions of "immunized" spleen cells. When the chambers were assembled, the spleen cells were forced to the periphery of the tumors. Here, the interaction between target cells and lymphocytes began. But, a few days later, tumor cells and lymphocytes, away from the zone of contact between these two types of cells, were also found degenerating.

These observations suggested that, when lymphocytes contacted homologous cells to which they had been "immunized," both types of cells died and diffusible substances were released which killed more nearby cells of these two types. Experiments are in progress to investigate this matter further.

Discussion

There appears to be general agreement on the following points concerning the deaths of homografts of both normal and malignant cells (*12*). The destruction of the grafts is immunologic in the sense that the responsible agent of the host is acquired after grafting. This is indicated by the fact that a second, similar graft is usually killed more rapidly than the first. The immunity is general rather than localized at the site of the graft. The genetic relationship between the cells of the graft and host are very important in determining the results of grafting. Homograft immunity is generally similar to immunity to bacteria or to foreign proteins except that circulating antibodies usually cannot be demonstrated and the immunity cannot be passively transferred by means of serum. The host cells which accumulate in and around homografts are predominantly lymphocytes.

There has been uncertainty as to the role, if any, of the lymphocytes in the destruction of homografts. This is illustrated by the following recent statement of Medawar (*3*):

"As Loeb and Murphy have long since insisted, the infiltration of tissue homografts by lymphocytes is a regular accompaniment to their break-

down. What are they doing? *Are they the seats of hypothetical homotoxins, or the vehicles by means of which antibodies are brought to the neighborhood* [italics ours]? Or is their presence merely a byproduct of the general inflammatory changes that accompany tissue destruction?"

Destruction of Target Cells on Contact With "Immunized" Cells in Vivo

The most important of the experimental results presented in this paper may be summarized as follows: Target cells in diffusion chambers impenetrable to cells survived in homologous hosts previously immunized to the target. Since similar targets did not survive when the hosts were heterologous and previously immunized, it may be presumed that such chambers were permeable to antibodies. Other target cells did not survive in more porous chambers penetrable by host leukocytes and macrophages when the chambers were placed in immunized homologous hosts. In addition, target cells combined in cell-impenetrable chambers with "target-immunized" spleen cells were destroyed even in hosts isologous to the target cells.[15] Control target cells exposed to or combined with homologous "nonimmunized" or with isologous cells remained in good condition during the same periods of time. It appeared that the "immunized" homologous cells responsible for the destruction of target cells were lymphocytes, although it is recognized that more data are needed on this point. For convenience in the following discussion, the cells responsible for this destruction will be referred to as lymphocytes.

These results imply that in homograft reactions the destruction of the graft is the result of close association, and probably of *contact*, between cells of the graft and host. There is, in these experiments, no evidence that such destruction *requires* circulating noncellular antibodies developed in response to the graft, if such antibodies do exist.

This concept is not new. As will be cited, several authors have presented evidence that the host cells, particularly the lymphocytes, which come in contact with homografts are of importance in their destruction. The experiments herein reported had certain technical advantages, however, over previous ones in analyzing the destructive mechanism.

In the present experiments, genetic relations between host and implant cells were controlled by use of inbred animals, and normal tissues as well as tumor tissues were used as target cells. The diffusion chambers employed were helpful in several ways. They enabled us to test over a prolonged period *in vivo* the influence of diffusible substances from the immunized host in the absence of host cells. This separation was not clearly accomplished in the past. Through the fortunate fact that one type of filter permitted passage of host leukocytes and macrophages, the influence of these cells could be studied (in the presence, of course, of whatever diffusible substances may have been present). It was possible to combine different types of cells in chambers with fair certainty that they

[15] "Immunized" peritoneal exudate cells (lymphocytes and macrophages) have been similarly tested on a small scale and appear to be equally destructive.

would remain in close association. Washed "immunized" or control lymphocytes could be combined with targets in chambers placed in hosts *isologous* to the target cells. It is unlikely that host factors could cause immunologic injury to these target cells. Finally, it was easy to locate for histologic or biologic study cells in chambers after intraperitoneal implantation.

The following authors have performed experiments the results of which favor the concept that homografts are destroyed by contact with "immunized" lymphocytes.

Lymphocytic destruction of homografts, according to Woglom (*13*), was first proposed by Wade (*14*) in 1908. In 1912, Da Fano (*15*) thought that the "intimate connection" of lymphocytes "with the development of immunity" to homografts was practically certain. Loeb (*16*) correlated directly the intensity of the lymphocytic reaction in the vicinity of grafts with the genetic disparity between the graft and host. Apparently he did not ascribe to the lymphocyte any direct role in graft destruction. Murphy (*17*) claimed that homografts are directly destroyed by the lymphocytes in their vicinity. Of particular significance is his finding that tumor homografts implanted alone in the brains of immunized hosts usually survived, but grafts combined with pieces of host spleen often did not survive. Since combination with host spleen in nonimmunized animals had less inhibitive effect, this inhibition was partially a function of immunity.

Substantiating Murphy's results and earlier reports, Woglom (*18*) reported that tumor grafts could be inhibited in rats if a paste of spleen cells from other rats immunized to the tumor was inoculated with the tumors. Spleen cells from nonimmunized rats had no effect. Also, Woodruff and Woodruff (*19*) have recently stated that homografts of thyroid tissues were injured when implanted together with host spleen into the anterior chambers of the eyes of guinea pigs but not when implanted with spleen from the donor of the thyroid tissue.

Kidd (*20*) has emphasized the intimate association of host lymphocytes with the cells of a degenerating tumor homograft as seen in sections.

Mitchison (*21*) has found that immunity to a homologous tumor can be passively transferred with minced lymph nodes from immunized mice but not by serum from these mice. Chase's (*22*) finding that skin sensitivity to tuberculin or to simple organic chemicals can be passively transferred by living lymphocytes, but not by serum, led Mitchison to postulate that these sensitivities and homograft immunity may have a common mechanism.

However, in all of the experiments just cited, only Kidd (*20*) and Mitchison (*21*) used inbred animals and specific tumors. Also, in the experiments in which grafts were combined with spleen, it is not possible to be certain that the hosts had nothing to do with the inhibition or destruction of the grafts since most of the hosts were homologous to the grafts. In these combinations of tissues with spleen and in Mitchison's passive transfer of homograft immunity by lymph nodes, the spleen and lymph

node cells were not washed and it may be that the effects observed were not due to the cells but to antibodies carried along in the extracellular fluids with the cells. These limitations were avoided in the experiments herein reported.

Turning to serologically demonstrable types of immunity, there is considerable evidence that here too lymphocytes play a role but it has also been claimed that plasma cells and the reticuloendothelial system are active. The conflicting evidence in this field has been well reviewed by McMasters (*23*).

There is little evidence against the hypothesis that lymphocytes are involved in homograft destruction. Darcy (*24*) has studied histologic sections of homografts disintegrating in rabbits. He cites clear evidence that plasma cells were not involved but his evidence with regard to the role of the lymphocyte is far less conclusive and he only tentatively suggests that they are not involved. Dempster (*25*) has said, ". . . homotransplanted skin disintegrates without any significant mesenchymal cell reaction." If it could be clearly shown that homografts frequently disintegrate without any lymphocytic reaction or even that only a few lymphocytes were present, considerable doubt would certainly be cast on the concept that homografts are destroyed by lymphocytes. However, this is not an easy matter to investigate. Simply studying fixed material without experimental manipulation, as Dempster did, is not adequate, as he himself says. A paucity or even total absence of lymphocytes at the site of a dying homograft at any one time does not prove that they were never there in considerable numbers. They could have wandered away, transformed into other types of cells, or had disintegrated before the tissue was fixed.

Contact of Target Cells With "Immunized" Cells in Vitro

Since in diffusion chambers the destructive action of "immunized" spleen cells on targets was independent of the strain or even of the species of the hosts in which the chambers were placed, the failure to obtain similar results *in vitro* is something of a puzzle. It seems unlikely that this was due to the fact that our cultures were maintained on heterologous media since Medawar (*26*), performing similar experiments, used completely homologous media and also obtained negative results. In some of his experiments he "glued," by means of plasma clots, pieces of "immunized" lymph node or spleen to target-cell fragments of skin and the combined tissues were then cultured. In a few days epidermal cells migrated around and tended to encapsulate the lymph nodes or splenic fragments. It appeared that there was contact between the two homologous tissues but, nonetheless, the target epidermal cells remained healthy. This occurred even when the fluid phase of the cultures was "immune" serum.

It is not considered that these negative results weaken the concept that homografts are destroyed by contact with "immunized" lymphocytes. Conditions *in vitro* may have been unfavorable in a number of ways.

It may be that the lymphocytes *in vitro* were unhealthy. However, it is to be noted that in our experiments, though most of the lymphocytes were dead after 5 days, many were certainly alive for 3 days and, during this period, similar cell combinations in diffusion chambers *in vivo* led to clearly positive results. Moreover, in his experiments Medawar took precautions to preserve the lymph node and spleen cells in good condition by supplying them with adequate amounts of oxygen.

The large volume of extracellular fluid necessary to maintain a given number of cells *in vitro* (far greater than that required *in vivo*) may have resulted in too great a dilution of substances released by cells and necessary in a certain critical concentration for a positive result.

Or perhaps the lysis of homograft cells involves the release from "immune" lymphocytes of antibodies which are active only in the presence of complement. Complement is required *in vitro* for the lysis of mammalian cells by antisera prepared by inoculation of such cells in a different species (*27*), for the lysis of erythrocytes or bacteria by antibodies (*28*), and for the lysis of lymphocytes from tuberculin-sensitive animals by tuberculin (*29*). Moreover, complement is quite unstable outside the body (*28*). Probably very little complement was initially present in the nutrient media used in our *in vitro* experiments. In Medawar's tissue-culture experiments it could have deteriorated by the time the target epidermal cells had migrated around to contact the attached "immunized" lymph node or spleen fragments.

In contrast to our negative results and those of Medawar *in vitro*, Kidd (*20*) has found that the subsequent growth of a specific mouse tumor in inbred isologous mice can be inhibited by prior incubation for 1 to 2 hours at 37° C. of the tumor with minced lymph nodes from immunized mice. Control lymph nodes from nonimmunized mice had no effect. This work has been published in detail.

Death of "Immunized" Lymphocytes on Contact With Target Cells

It appeared in our experiments that "immunized" lymphocytes died in attacking target cells. The death of lymphocytes at the site of degenerating homografts has been noted by others (*15*, *24*, *30*). These observations may explain the finding of Dempster (*25*) that the host's cellular reaction to skin homografts was insignificant (quoted above) and that of Da Fano (*15*) who said that there was no accumulation of lymphocytes about a second homograft in an immunized host. It is interesting to note that lymphocytes from animals sensitized to tuberculin also die when tuberculin is added to them *in vitro* (*29*).

If each "immunized" lymphocyte in a diffusion chamber could have destroyed only so many target cells, then the number of such lymphocytes contacting the target cell and the initial number and rate of multiplication of target cells would have been of great importance in determining whether the latter cells survived. Lymphocytes are not ordinarily present in large numbers in the sites where grafts are placed. Hence anything which

causes these cells to accumulate near a graft could be of importance. A tumor homograft could survive permanently in a foreign host and even grow there if not all its cells were killed by the initial lymphocyte accumulation. The tumor cells could "keep ahead" of the "immunized" lymphocytes by mitosis. Once the tumor was vascularized and the initial inflammatory reaction had subsided, the tumor could survive and grow indefinitely provided there was no massive accumulation of "immunized" lymphocytes.

Summary

To investigate whether homografts in mice are destroyed by circulating antibodies, by host cells, or by both, mouse tissue "target" cells incorporated in diffusion chambers were implanted intraperitoneally in isologous or homologous mice or in rats. Some of the homologous and heterologous hosts had previously been immunized to the target cells by inoculation of spleen from mice of the target cell strain of origin. One type of chamber was designed so that host cells were excluded ("cell-impenetrable") and another so that host leukocytes and macrophages entered ("cell-penetrable").

In "cell-impenetrable" chambers, target cells survived in isologous or homologous hosts, even when the latter had been previously immunized to the target cells. Target cells in such chambers also survived for at least a week in nonimmunized rats but not in immunized rats, indicating that the chambers were permeable to antibodies.

The target-cell contents of "cell-penetrable" chambers survived in isologous mice. The target cells usually survived for 2 weeks in similar chambers in homologous mice, but sometimes died later. In homologous mice previously immunized, however, the target cells usually died within 2 weeks.

Using as hosts mice isologous to the target cells, the combination of washed spleen cells from target-immunized mice with target cells in "cell-impenetrable" chambers tended to kill the target cells within a week. In controls, addition of isologous or homologous "nonimmunized" spleen cells had no effect during the first week, although later the target cells exposed to homologous spleen cells sometimes died.

Observations were made which suggested that: 1) the "immunized" cells which were responsible for the death of target cells were lymphocytes; 2) when these lymphocytes contacted cells to which they had been "immunized," both types of cells died; and 3) such death was followed by the release of diffusible substances (antibodies and antigens?) which killed nearby cells of both types.

The simplest interpretation of these results would imply that homografts in mice are usually destroyed by close association with "immunized" host cells (probably by *contact with lymphocytes*) rather than by exposure to circulating noncellular antibodies. These results are compatible with the hypothesis that antibodies to homografts are transported by lymphocytes.

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PLATE 148

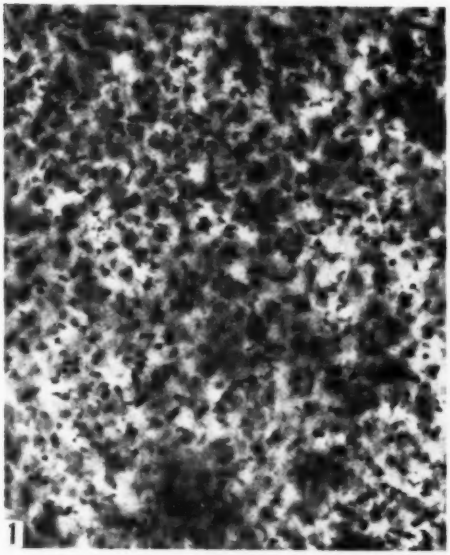
All photomicrographs are of whole mounts of diffusion chambers fixed with Zenker-formol and stained with hematoxylin.

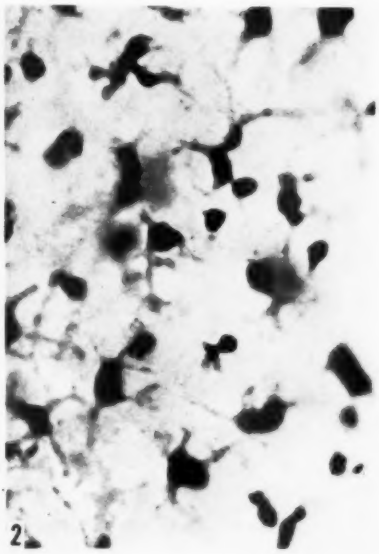
FIGURE 1.—Cells inside a 150μAA filter after 25 days in a C mouse. The photograph was taken by focusing about half way through the thickness of the filter. × 250

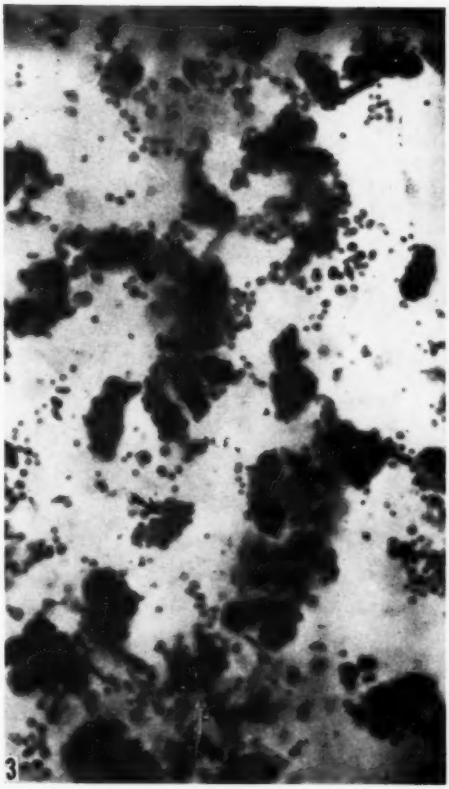
FIGURE 2.—Host cells which have passed through a 150μAA filter and are spreading out inside the diffusion chamber. Macrophages and leukocytes are seen. Three days in a C × C3H host. × 1,000

FIGURE 3.—A suspension of "epidermal" cells fixed immediately after preparation. The rounded masses of cells are hair follicle rudiments. One small sheet of basal cells, is present (*arrow*). The small individual cells are degenerating basal cells of the epidermis. A few large individual granular cells of the epidermis are present. × 250

FIGURE 4.—A spleen-cell suspension (C × C3H) in a diffusion chamber for 5 days in a C × C3H host. The cells are healthy and dividing. × 1,000







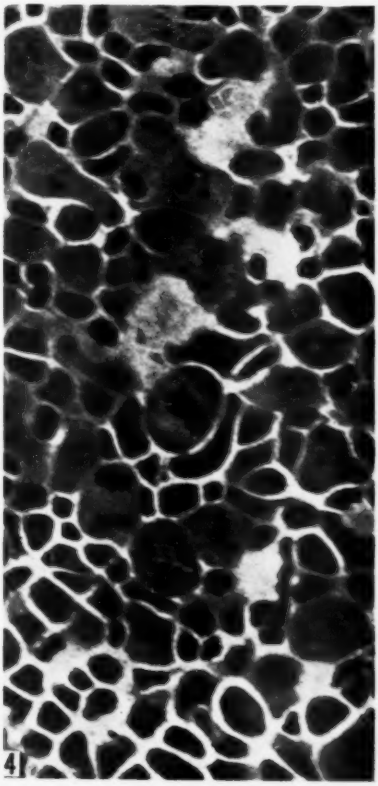


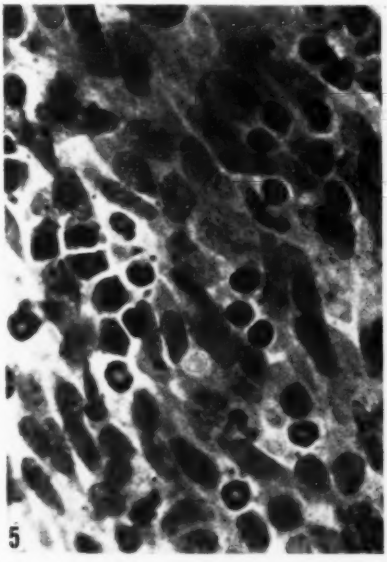
PLATE 149

FIGURE 5.—The mesenchyme of a C × C3H lung rudiment in a diffusion chamber after 7 days in a Holtzman rat. The cells are healthy and dividing. Hematopoiesis is occurring. The chamber did not admit host cells. × 1,000

FIGURE 6.—Same as figure 5, but the rat had been previously immunized by C × C3H spleen. The tissue is necrotic—presumably because antibodies entered the chamber. × 1,000

FIGURE 7.—The edge of a sheet of epidermal cells (C × C3H) in a chamber after 4 days in a Holtzman rat. The chamber did not admit host cells. The epidermal cells are healthy and dividing. × 250

FIGURE 8.—Same as figure 7, but the rat had been previously immunized by C × C3H spleen. The cells are necrotic. × 250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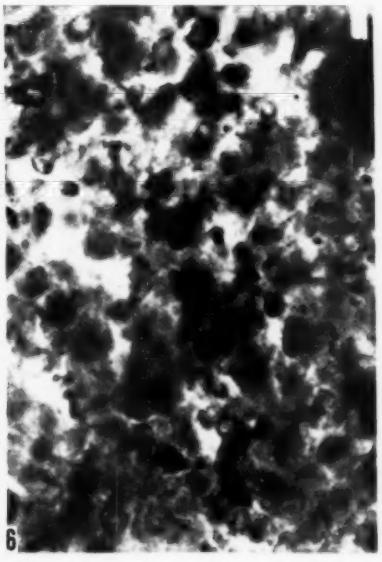
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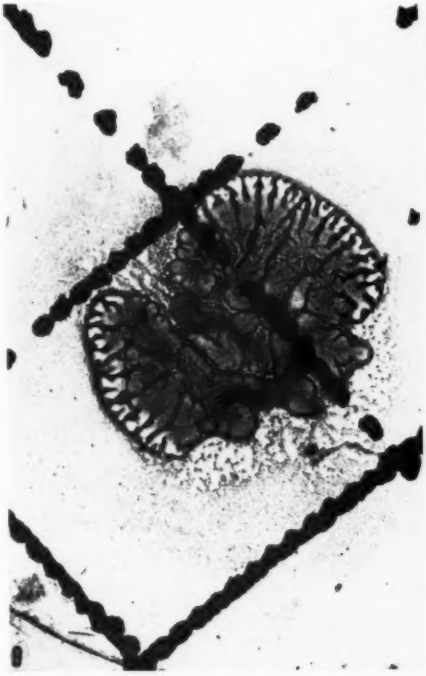
PLATE 150

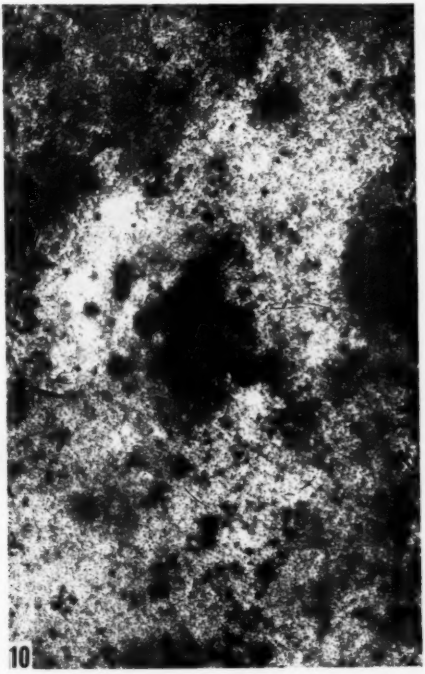
FIGURE 9.—A lung rudiment (C × C3H) in a cell-impenetrable diffusion chamber after 6 days in a C57BL mouse previously immunized by C × C3H spleen. The lung is healthy, growing, and differentiating. The results were similar with other lungs in such chambers in C × C3H or C57BL mice. The black lines are grids printed on the filter. (This rudiment is shown at higher magnification in figure 13.) × 15

FIGURE 10.—Same as figure 9, but the chamber admitted host cells. The rudiment has been destroyed. × 15

FIGURE 11.—Same as figure 10, but the host was a nonimmunized C57BL mouse. The lung in figure 10 was destroyed because of the entrance of "*immunized*" host cells—not because they were homologous cells. The rudiment in figure 11 is significantly smaller than those in chambers which did not admit host cells, as in figure 9. × 15

FIGURE 12.—Same as figure 11, but the host was a C × C3H mouse. The admission of host cells to a chamber from any host decreases the size of a rudiment, as compared with the size when these cells are excluded. The difference in the size of the rudiment in this figure and that in figure 11 is not significant. × 15

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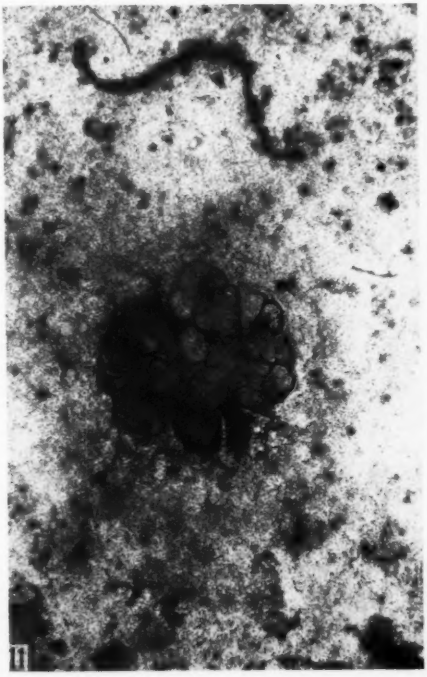
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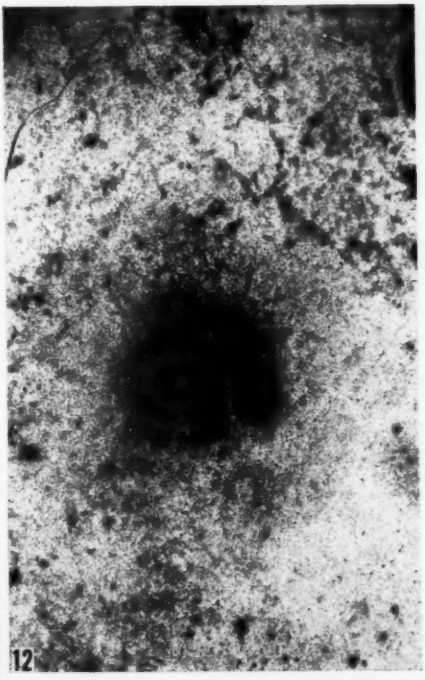
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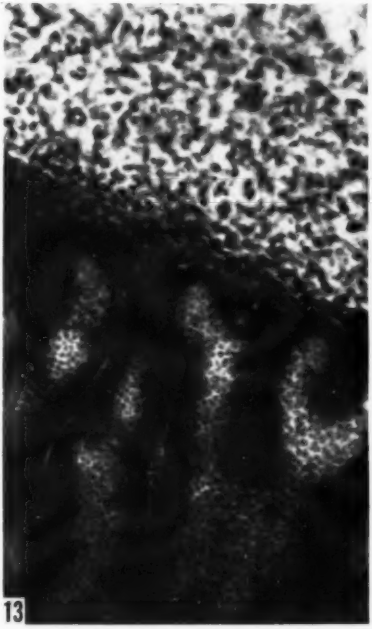
PLATE 151

FIGURE 13.—A higher magnification of the lung rudiment in figure 9. Epithelium is at the *bottom;* mesenchyme, at the *top*. The cells are all healthy. × 250

FIGURE 14.—A lung rudiment (C × C3H) combined with C × C3H spleen cells in a cell-impenetrable diffusion chamber after 5 days in a C × C3H mouse. The rudiment is healthy, growing, and differentiating. Lung mesenchyme is spreading through the spleen cells. × 15

FIGURE 15.—Same as figure 14, but the spleen cells were from a C57BL mouse. The result is the same. × 15

FIGURE 16.—Same as figure 14, but the spleen cells were from a C57BL mouse previously immunized by C × C3H spleen. The epithelium of the rudiment has not grown or differentiated and about 30 percent of these cells are degenerating. The mesenchyme of the lung has been destroyed. Many of the spleen cells about the rudiment are dead. × 15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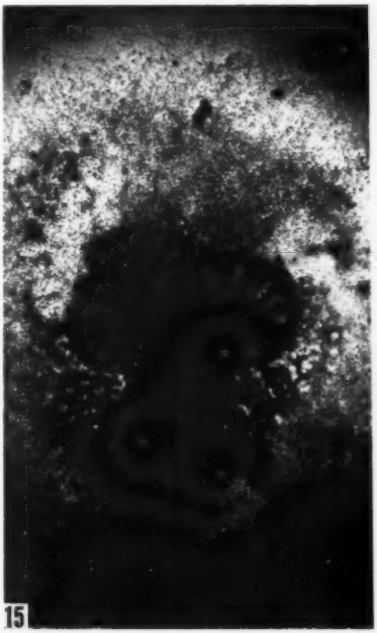
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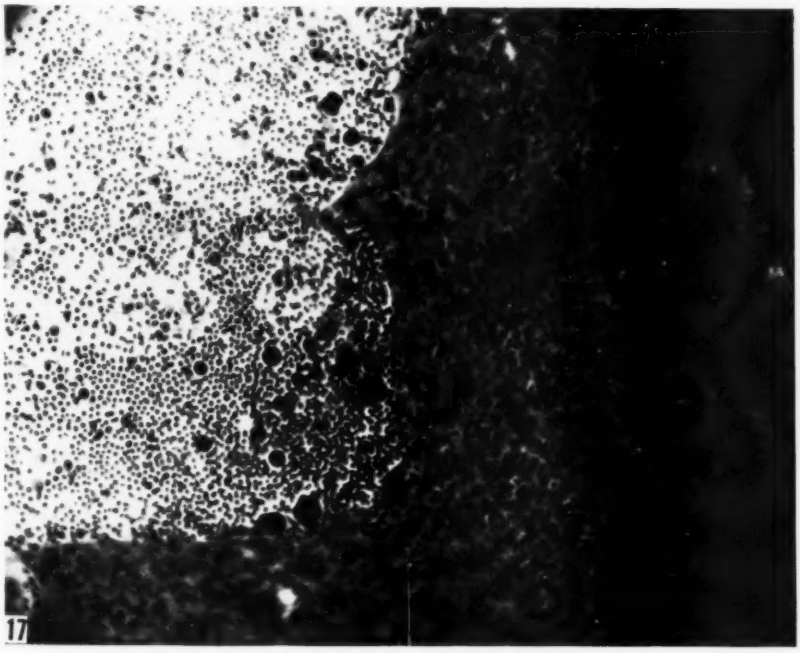
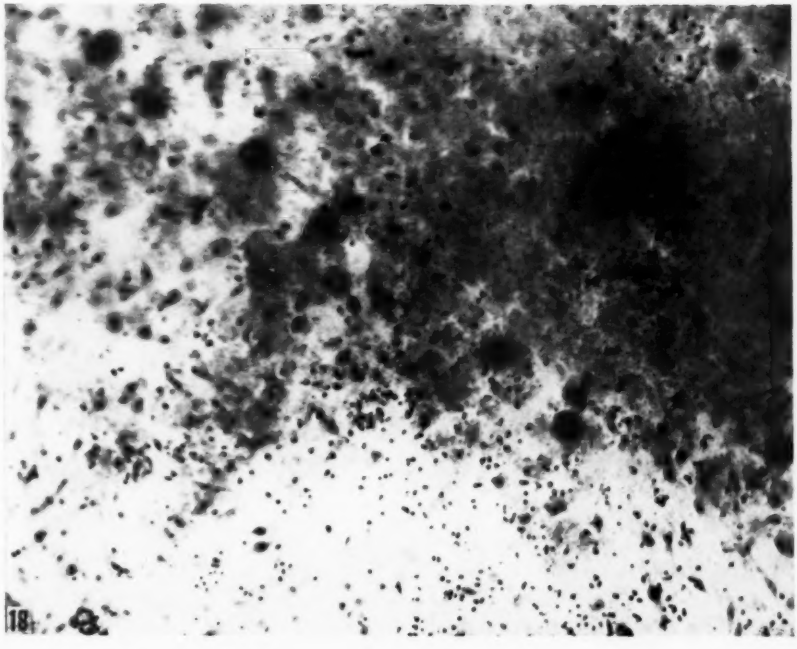
PLATE 152

FIGURE 17.—C3HBA mammary adenocarcinoma (a C3H tumor) combined with C3H spleen cells in a cell-impenetrable diffusion chamber after 14 days in a C3H host. The edge of the advancing healthy tumor sheet is in the center of the field. Note the infiltration of tumor on the *right* with leukocytes from the spleen. Spleen cells are on the *left*. The very large cells here are megakaryocytes. × 250

FIGURE 18.—Same as figure 17, but the spleen cells were from a C mouse previously immunized by C3H spleen, and the chamber was in a C3H host 7 days. The tumor cells are dead. Most of the spleen cells are dead. × 250





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